# ANNUAL REVIEW OF MICROBIOLOGY

CHARLES E. CLIFTON, *Editor*
*Stanford University*

SIDNEY RAFFEL, *Associate Editor*
*Stanford University*

MORTIMER P. STARR, *Associate Editor*
*University of California*

VOLUME 12

1958

ANNUAL REVIEWS, INC.
PALO ALTO, CALIFORNIA, U.S.A.

ANNUAL REVIEWS, INC.
PALO ALTO, CALIFORNIA, U.S.A.

© 1958 BY ANNUAL REVIEWS, INC.
ALL RIGHTS RESERVED

*Library of Congress Catalog Card Number: 49-432*

FOREIGN AGENCY

Maruzen Company, Limited
6, Tori-Nichome Nihonbashi
Tokyo

PRINTED AND BOUND IN THE UNITED STATES OF AMERICA BY
GEORGE BANTA COMPANY, INC.

# PREFACE

The words serving as an introduction to the chapter, "Bacterial Viruses," by W. Weidel, remind us of the ever-changing concepts in microbiology. The diligent efforts of our contributors, to whom we express our appreciation, serve to keep us informed of the progress being made in the various fields and to present new problems which, in effect, say with Dr. Weidel, "it ain't necessarily so!"* Today's concept may or may not become tomorrow's fact.

The Editorial Committee desires to express its gratitude to those who have made suggestions for topics and for authors, and to invite further suggestions for future volumes.

We wish to express gratitude also to Dr. Pierre Grabar for his reviews of the Russian Literature for the past three years. The increasing availability of abstracts and translations reduce the need for future reviews of this nature.

At this time we extend our thanks to Dr. Roger Stanier for his efforts as Associate Editor, and to Dr. Perry Wilson for his valuable services on the Editorial Committee. We are pleased to announce the appointment of Dr. Mortimer Starr as the new Associate Editor, and Dr. Robert Hungate as Dr. Wilson's successor.

Once again, we express our deepest appreciation for the valuable assistance and cooperation of the office staff of Annual Reviews, Inc., acknowledging, in particular, the extremely capable efforts of Miss Beryl Daniel as editorial assistant.

| | |
|---|---|
| S.P.C. | S.R. |
| C.E.C. | M.P.S. |
| C.A.E. | T.M.S. |
| R.E.H. | W.W.U. |

* Copyright difficulties prevent us from using the appropriate bars of music as originally suggested by Dr. Weidel.



THE UNIVERSITY LIBRARIES OF GEORGIA

# TOPICS AND AUTHORS
# ANNUAL REVIEW OF MICROBIOLOGY
# VOLUME 13 (1959)


Antibiotics in Food Preservation, *L. Farber*

Nonspecific Resistance to Infection, *M. Shilo*

Advances in Immunology, *A. Bussard*

Immunologic Tolerance, *M. W. Chase*

Biosynthetic Aspects of Metabolism, *R. B. Roberts and I. Z. Roberts*

Mechanisms of Terminal Respiration, *H. L. Kornberg*

Fermentative Metabolism, *J. C. Rabinowitz*

Bacterial Spores, *J. F. Powell*

Biochemical Cytology of Microorganisms, *P. Mitchell*

Unicellular Clocks, *J. W. Hastings*

Genetics and Cytology of Chlamydomonas, *R. P. Levine and W. T. Ebersold*

Bacterial Genetics (With Particular Reference to Genetic Transfer in the Enterobacteriaceae), *P. E. Hartman*

Phage Genetics, *C. Bresch*

Applications of Tissue Culture in Microbiology, *H. E. Swim*

Heterocaryosis, *K. C. Atwood*

Complex Loci in Microorganisms (With Particular Reference to Sequential Steps in Biosynthesis), *M. Demerec and P. E. Hartman*

Newer Antibiotics, *W. F. Verwey*

Nematodes in Plant Disease, *M. J. Fielding*

Plant Disease Resistance, *H. L. Barnett*

Bacteria as Plant Pathogens, *M. P. Starr*

Sulfate Reduction by Bacteria, *J. R. Postgate*

Fine Structure of Virus-Infected Cells (Structure of Both Viruses and Cells), *H. M. Rose and C. Morgan*

New Viruses and Virus Diseases of Man, *M. Schaeffer*

Biology of Tetrahymena, *A. M. Elliott*

Physiology and Development of the Lower Fungi, *E. C. Cantino*

# CONTENTS


| | PAGE |
|---|---|
| BACTERIAL PROTOPLASTS, *C. Weibull* | 1 |
| BACTERIAL VIRUSES (WITH PARTICULAR REFERENCE TO ADSORPTION /PENETRATION), *W. Weidel* | 27 |
| NEWLY RECOGNIZED RESPIRATORY TRACT VIRUSES, *R. J. Huebner, W. P. Rowe,* and *R. M. Chanock* | 49 |
| THE USE OF BACTERIA GROWN *In Vivo* FOR STUDIES ON THE BASIS OF THEIR PATHOGENICITY, *H. Smith* | 77 |
| PARASITIC DISEASES OF MAN (Recent), *E. C. Faust* | 103 |
| THE CONTROL AND ERADICATION OF ANIMAL DISEASES IN THE UNITED STATES, *W. A. Hagan* | 127 |
| METABOLISM OF CARBOHYDRATES AND RELATED COMPOUNDS, *S. R. Elsden* and *J. L. Peel* | 145 |
| INORGANIC NITROGEN METABOLISM, *A. Nason* and *H. Takahashi* | 203 |
| MICROBIAL NUTRITION, *B. M. Guirard* | 247 |
| NUTRITION AND ECOLOGY OF PROTOZOA AND ALGAE, *L. Provasoli* | 279 |
| BACTERIAL GENETICS, *A. W. Ravin* | 309 |
| GENETICS OF FUNGI, *H. E. Wheeler* | 365 |
| REVIEW OF THE MICROBIOLOGICAL AND IMMUNOLOGICAL LITERATURE PUBLISHED IN 1957 IN THE U.S.S.R., *P. Grabar* | 383 |
| ANTIBIOTICS IN THE CONTROL OF PLANT DISEASES, *W. J. Zaumeyer* | 415 |
| MASKED VIRUS INFECTION IN PLANTS, *E. M. Hildebrand* | 441 |
| PLANT TISSUE CULTURES OPEN A BOTANICAL FRONTIER, *A. J. Riker* and *A. C. Hildebrandt* | 469 |
| FACTORS DETERMINING THE PATHOGENICITY OF STAPHYLOCOCCI, *J. E. Blair* | 491 |
| MICROBIOLOGICAL ASPECTS OF RADIATION PRESERVATION OF FOOD, *C. F. Niven, Jr.* | 507 |
| CHEMICAL DISINFECTANTS, *C. R. Phillips* and *B. Warshowsky* | 525 |
| INDEXES | 551 |




5-59

Annual Reviews, Inc., and the Editors of its publications assume no responsibility for the statements expressed by the contributors to this *Review*.

# BACTERIAL PROTOPLASTS[1,2]

By Claes Weibull[3]
*Central Bacteriological Laboratory of Stockholm City, Stockholm, Sweden*

During the last fifteen years the organization of the bacterial cell has been mainly studied in three different ways: by light microscopy, by electron microscopy, and by disintegrating cells and characterizing the disintegration products.

Perhaps the most remarkable result of the light microscope studies has been the clear demonstration of nuclear equivalents in bacteria [Robinow (1, 2)]. Electron microscope investigations on whole bacteria have increased our knowledge of the outer morphology of these organisms considerably. This is evidenced, for example, by the systematic studies on bacterial flagella performed by Houwink & van Iterson (3) and van Iterson (4). Very valuable information concerning the internal structure of bacterial cells has been obtained by preparing sections of bacterial cells and studying these sections under the electron microscope. In this way, the presence of several well-defined structures in the bacterial cell has been elegantly demonstrated, viz., the cell wall and the cross-septa originating from the wall, a granular cytoplasmic ground substance and nuclear bodies [works by Chapman & Hillier (5) and other investigators].

A more or less drastic disintegration of bacterial cells followed by a fractionation of the resultant products has proved useful for studying the organization of these cells. In this way, beginning with the studies of Weibull (6) and Salton (7), the structure and chemical nature of bacterial flagella and cell walls have been investigated. Using a similar technique, Schachman, Pardee & Stanier characterized the bacterial protoplasm in terms of its macromolecular constituents (8).

As in most scientific fields, the results obtained from studies on the organization of the bacterial cell have not always been in good agreement. On the contrary, the field of bacterial anatomy has been a very controversial one during the last few years. The points of disagreement have, however, mainly concerned the interpretation of results obtained by using only one of the above-mentioned techniques. In the cases where several

[1] The survey of literature pertaining to this review was completed in December, 1957. Investigations on protoplasts reviewed in references (31, 50, and 96) are treated somewhat less in detail than more recent studies.

[2] The following abbreviations are used: DAP (diaminopimelic acid); DNA (deoxyribonucleic acid); EDTA (ethylenediaminetetraacetic acid, versene); RNA (ribonucleic acid); "Tris" (tris(hydroxymethyl) aminomethane).

[3] The author wishes to thank Dr. E. H. Cota-Robles for helpful discussions and criticism of the manuscript.

methods of investigation have been used to check the experimental results, less conflicting opinions have been expressed. Thus, most workers seem to agree as to the existence of a rigid cell wall, a cytoplasmic membrane, a granular cytoplasmic ground substance, and nuclear bodies in at least most bacterial cells.

In particular, the fact that the bacterial cell wall is a well-defined structure differentiated from the internally located protoplasmic constituents has been demonstrated very clearly by plasmolysis experiments [Fischer (9); Ellis (10); Swellengrebel (11); Garbowski (12); Vahle (13); Raichel (14) Knaysi (15); Elo (16)], by investigations concerning the structure and chemical composition of the wall [reviewed by Salton (17)] and by cytological studies [reviewed by Knaysi (18)]. In addition, Salton has shown, using isolated cell walls of *Micrococcus lysodeikticus,* that the cell wall is the substrate of the lytic agent lysozyme (19). The same conclusion was drawn by Tomcsik & Guex-Holzer (20) and by Weibull (21) who worked with whole cells of *Bacillus megaterium.* Tomcsik & Guex-Holzer "stained" the cells with specific antibodies and followed the change of the cell shape and appearance which was caused by the action of lysozyme. It appeared that the originally cylindrical protoplasts became separated from the surrounding, disintegrating cell wall and assumed a spherical shape. Shortly afterwards they became transparent and eventually changed into amorphous debris. Weibull studied, independently of Tomcsik & Geux-Holzer, the lytic action of lysozyme on *B. megaterium,* measuring the turbidity of bacterial suspensions and observing microscopically cells that had not been treated with antibodies. He observed that the turbidity of suspensions prepared with dilute phosphate buffers rapidly dropped to a very low value as a consequence of the addition of lysozyme. If, however, sucrose or polyethylene glycol was present in the suspension, the drop in turbidity proceeded initially as fast as in the absence of these compounds but stopped at a much higher level. A microscopic investigation of the reaction mixture revealed that spherical bodies having the same general appearance as the intact bacterial cells, except for the shape, had been formed. These bodies seemed to be quite stable for a number of hours or days when stored in the stabilizing medium but lysed instantaneously when transferred to distilled water or dilute buffers. Chemical and physiological investigations strongly suggested that the spherical bodies consisted of cells deprived of their walls but otherwise essentially intact. These bodies were named protoplasts. They have attracted the interest of microbiologists because of their use as valuable tools for structural, metabolic, and physiological studies.

## Structure of Bacterial Protoplasts and Related Forms. Terminology

Gravimetric analyses of the fluid surrounding lysozyme-treated cells of *B. megaterium,* strain KM, and untreated controls revealed that substances corresponding to at least 20 per cent of the bacterial dry weight were liber-

ated when the cells were treated with lysozyme in the presence of sucrose [Weibull (21, 22)]. On the other hand, no ultraviolet-absorbing compounds (21) or inorganic phosphorus (22) left the cells. The spherical bodies remaining after the lysozyme treatment did not absorb bacteriophages in contrast to isolated cell walls (21). In combination with earlier findings concerning the dry weight [Cooper, Rowley & Dawson (23)] and chemical nature [Salton (7)] of the bacterial cell wall, these data strongly suggested that the cell wall of the bacterium investigated had been completely digested. This has been confirmed in various ways. Thus, Salton showed by means of electron microscopy that isolated cell walls of *B. megaterium*, strain KM, completely disintegrated when treated with lysozyme (24). Welshimer obtained the same results when studying cell wall fragments of strain 899 of the same organisms by means of phase contrast microscopy (25). Weibull studied bacteria suspended in India ink (26). In such a medium the cell wall appeared as a bright boundary at the periphery of the intact cells. This boundary disappeared when the cells were treated with lysozyme in the presence of sucrose. Salton noted that the cell wall of boiled bacteria was removed by lysozyme, leaving the coagulated protoplasmic constituents in the form of small rods (27). Tomcsik & Guex-Holzer (28), Vennes & Gerhardt (29), and Vennes (30) showed that cells of *B. megaterium*, strains KM and M treated with lysozyme in sucrose solutions, did not react with antisera prepared against isolated cell walls. McQuillen noted the absence of DAP in cells treated in the same way (31). According to Work (32), this amino acid is found mainly in the cell wall fraction of bacterial cells. Weibull & Thorsson studied the action of lysozyme on cells of *B. megaterium*, strain M, using electron microscopy of thin bacterial sections (33). They showed that lysozyme treatment in the presence of a stabilizing agent has no major effect on the internal organization of the cells. Thus, a granular cytoplasmic ground substance and nuclear equivalents were observed both before and after this treatment. On the other hand, the wall structure characteristic of intact cells totally disappeared as a consequence of lysozyme action.

A number of investigations, to be dealt with in more detail in a following section, have shown that the removal of the cell wall from *B. megaterium* cells does not necessarily affect very much the biochemical and biological capabilities of the organism. This must imply that the organization of the bacterial protoplast may be uninfluenced by the presence or absence of the cell wall.

In considering the available evidence it seems firmly established that the cell wall of the above-mentioned strains of *B. megaterium* can be completely and selectively removed from the cells by means of lysozyme, provided that a stabilizing agent, preventing further disintegration of the cell structure, is added to the medium around the cells. Thus, it seems that we are justified in designating as protoplasts the remaining, spherical cell bodies. It is true that, by using this nomenclature, bacterial protoplasts may refer to two, not quite identical things, viz., the protoplasmic substances when free or when

located within the protective cell wall. So far, however, little confusion seems to have arisen from this usage of the word protoplast. Stähelin (34) and Colobert (35) have suggested the term "gymnoplast" for free protoplasts. The present author feels that this suggestion deserves more attention than that actually paid to it so far.

A number of papers describing the formation of spherical bodies similar to *B. megaterium* protoplasts, but prepared from other bacterial species, have been published. It is doubtful, however, whether the cell wall was completely removed from the cells in all of these studies. Often the only evidence presented, suggesting that this was accomplished, has been the spherical shape of the bodies and the fact that they lyse in the absence of a stabilizing agent in the medium (cf. below). Only a partial degradation or "softening up" of the cell wall could be sufficient, however, to cause these changes.

Oparin *et al.* (36), Mitchell & Moyle (37), Bridoux & Hanotier (37a), Britt & Gerhardt (38), Britt (39), and Few *et al.* (40) have described the preparation of osmotically fragile cells of *M. lysodeikticus* and *Sarcina lutea* by means of lysozyme treatment. It seems highly probable that most of the cell bodies thus obtained were free of wall material since isolated cell walls of these organisms can be completely dissolved by lysozyme [Salton (19, 24)]. Colobert (35) and Wiame, Storck & Vanderwinkel (41) found, however, that strong saline solutions inhibit lysozyme action; therefore one could perhaps question the nature of the osmotically fragile cells obtained by Mitchell & Moyle (37) with the aid of 1-2 *M* NaCl as stabilizing agent.

Using lysozyme, Wiame, Storck & Vanderwinkel (41) and Mutsaars (42) converted *Bacillus subtilis* cells into spherical, osmotically fragile bodies. The electron micrographs presented by the former authors strongly suggest that the cell wall was completely dissolved by the lysozyme treatment. On the other hand, Salton noted that isolated cell walls of *B. subtilis* are only partly degraded by lysozyme (43). Perhaps these somewhat conflicting results could result from a variation in lysozyme sensitivity from one strain of *B. subtilis* to another. Tomcsik & Baumann-Grace reported such a variation in *B. megaterium* (44, 45).

Strange & Dark (46, 47, 48) found that extracts of disintegrated spores and partial autolysates of *Bacillus* spp. contained enzymes that lysed isolated cell walls of vegetative *Bacillus* cells. The same workers used these enzymes for the conversion of living *Bacillus cereus* and *B. megaterium* cells into spherical osmotically fragile bodies (48). These bodies may still have contained some wall material, however, since it does not seem conclusively established that isolated *Bacillus* cell walls are completely degraded by the enzymes used (46, 47).

According to Fischer (49), the protoplasmic constituents of a bacterium may sometimes be ejected from the cell and thus become separated from the cell wall. Later investigations [reviewed by Weibull (50)] have con-

firmed Fischer's observations. Stähelin demonstrated, for example, very clearly the ejection of the cell contents from a certain strain of *Bacillus anthracis* (34, 51). The ejected protoplasmic bodies were spherical in shape and stable in suitable media. Very probably, the ejected cell bodies were free from cell wall material since the walls remained in the medium as empty but rigid cylinders.

Mitchell & Moyle used partial autolysis as a method for obtaining osmotically fragile cells of *Staphylococcus aureus* (52). They concluded from electron micrographs and analytical data that autolysis results in the loss of an equatorial ribbon of the cell wall, leaving the cell contents as a protoplast capped by two hemispheres of cell wall material.

Recently, several investigators have described the conversion of *Escherichia coli* cells into spherical, osmotically fragile bodies using various experimental techniques [Zinder & Arndt (53); Mahler & Fraser (54); Fraser & Mahler (55); Rickenberg (56); Amano *et al.* (57); Panijel & Huppert (58); Liebermeister & Kellenberger (59); Lederberg (60, 61); Hahn & Ciak (62); Spizizen (63); Ben-Porat & Spiegelman (64); Nisman & Spiegelman (65); Chargaff, Schulman & Shapiro (66); Mitchell & Moyle (67); Meadow, Hoare & Work (68); Davis & Bauman (69); and McQuillen (70)]. In addition, similar studies have been performed on other Gram-negative bacteria [Lederberg (60); Repaske (71); Colobert (72); and Jeynes (73)]. Very little evidence is given, however, in these papers about whether the cell walls of the bacteria were completely dissolved or only party degraded. Amano *et al.* reported (57) that the enzymic extract used by them reduced the turbidity of suspensions of *E. coli* cell walls to rather low values. Light microscope studies revealed that the isolated walls were converted into an amorphous mass. Colobert (72) concluded from electron microscope studies that the wall was completely removed from the *Salmonella typhosa* specimens studied by him. Sectioned material was, however, not studied. McQuillen's finding (70), that some DAP was present in osmotically fragile *E. coli* cells, suggests that some cell wall material was still attached to these cells (32).

It is clear from the preceding paragraphs that, during the last few years, a number of investigators have described the conversion of various bacteria into spherical bodies sensitive to changes in the osmotic pressure of the surrounding medium. As has been pointed out, however, additional evidence besides osmotic fragility and change in shape is needed to demonstrate the complete removal of the cell wall from bacterial cells. Such evidence is lacking in many of the studies in this field (cf. above). In the author's opinion [cf. Mitchell & Moyle (52); Salton (74); McQuillen (70); and Colobert (72)], it seems undesirable to designate as protoplasts cell bodies to which some of the wall material is still attached. Mitchell & Moyle (52) suggested that, if the complete dissolution of the cell wall is in doubt, the term protoplast should be written within quotation marks. For want of a distinct, single-word term meaning "a bacterial cell osmotically fragile

because of a partial degradation of the cell wall" (the term "angioplast" suggested by Colobert (35) has hardly this meaning), this usage will be adopted in the following sections. Sometimes, however, the expression "protoplast-like bodies" will be preferred (written without quotation marks).

Mahler & Fraser (54) define bacterial protoplasts operationally (they use the word protoplast without quotation marks) as cells that have been altered to such an extent that (*a*) they are incapable of forming colonies; (*b*) they possess much of the organization of the cells and are definite morphological entities distinguishable microscopically; (*c*) they are osmotically fragile; and (*d*) they retain much of the synthetic capacity of the original cell. The present author is inclined to believe that this definition will cause more confusion than clarity.

To summarize this section: experimental data are available indicating that true, cell wall-free protoplasts have been prepared from cells of *B. megaterium* and *B. anthracis*. This is very probably true also of *B. subtilis*, *M. lysodeikticus*, and *S. lutea* cells. The spherical, osmotically fragile bodies that have been prepared from various other species should, awaiting further evidence, rather be designated as protoplast-like bodies or "protoplasts" since it cannot be excluded that part of the cell wall structure may still be attached to these bodies.

## Experimental Methods for the Preparation of Protoplasts and Protoplast-like Bodies

*Lysozyme treatment.*—The experimental technique varies from one bacterial species to another, being most straight-forward in the case of Gram-positive bacteria.

For the stabilization of *Bacillus* protoplasts, the media recommended by Weibull (21), i.e., buffered sucrose or polyethylene glycol solutions (pH 7), are still used. New media have, however, been introduced: Ringer's solution (75); 0.5 *M* phosphate, pH 7 to 7.8 (76, 77); 0.25 to 0.8 *M* NaCl (41, 77); 0.5 *M* sodium citrate (78); and 0.5 succinate (79). Wiame, Storck & Vanderwinkel (41) noted an inhibition of lysozyme activity by 0.25 to 0.5 *M* NaCl and thus used this medium only in metabolic experiments with protoplasts which had to be performed in the absence of sucrose. For the preparation of protoplasts a sucrose medium was used.

The requirements for protoplast stabilization seem to vary from one *Bacillus* species or strain to another. Tomcsik & Baumann-Grace showed (44, 45) that stable protoplasts can scarcely be obtained from *B. megaterium* strains which are characterized by large cells and great amounts of intracellular lipide granules. On the other hand, strains with smaller cells and less lipide inclusions were well-suited for the preparation of protoplasts.

When sucrose is used as the stabilizing agent for the preparation of protoplasts of *Bacillus* spp., concentrations of 0.2 to 0.6 *M* have been found effective in the case of *B. megaterium*, strain KM (21,29, 30, 77, 79 to 83), strain M (28, 84), and strain 899 (81). Weibull noted, however, some leak-

age of ultraviolet-absorbing substances from protoplasts of *B. megaterium*, strain M, in 0.5 *M* sucrose (84). According to Wiame, Storck & Vanderwinkel (41) and Mutsaars (42), protoplasts of *B. subtilis* require 0.5 *M* sucrose for their stabilization.

Protoplasts of *M. lysodeikticus* and *S. lutea* require for their stabilization a considerably higher osmotic pressure in the medium than *Bacillus* protoplasts. Thus, Mitchell & Moyle recommend the use of 1.2 *M* sucrose or 1 to 2 *M* NaCl (37). Other workers in the same field used media containing 0.8 to 1.0 *M* sucrose (36, 38, 39, 40).

Concentrations of lysozyme ranging from 10 to 1000 μg./ml. have been used for the preparation of protoplasts of Gram-positive bacteria. The optimum concentration depends, to some extent at least, on the cell concentration in the medium.

Evidently, protoplasts can be prepared from Gram-positive bacteria by the action of lysozyme alone at a pH around 7. This simple and physiologically mild treatment is apparently not successful in the case of Gram-negative bacteria. Zinder & Arndt described the conversion of *E. coli* cells to osmotically fragile bodies by the action of lysozyme alone, provided that the pH of the medium was adjusted to either 5 or 8 to 9 (53). As a rule, the conversion was performed at pH 9 since otherwise the "protoplasts" were badly agglutinated. "Tris" at a final concentration of 1 *M* served both as a buffer and a stabilizing agent. Other stabilizing media tried were: 0.6 *M* sucrose buffered with 0.1 *M* carbonate (pH 9), 0.5 *M* NaCl with carbonate buffer, and 0.3 *M* $NH_4Cl$ adjusted to pH 9 with NaOH. In the latter two media the cells did not assume a spherical shape but they did become osmotically fragile.

Zinder & Arndt used strains K12 and B of *E. coli*, grown in broth. Overnight cultures were diluted tenfold and incubated with aeration for 90 min. In this way cells just out of the exponential growth phase were obtained. Such cells were apparently better suited for the experiments than the more fragile, exponentially growing cells. The method described by Spizizen (63) for the preparation of osmotically fragile *E. coli* cells is rather similar to that of Zinder & Arndt.

Repaske found that cells of *E. coli*, *Azotobacter vinelandii*, and *Pseudomonas aeruginosa* can be lysed by lysozyme at pH 7.6 to 8 if EDTA is present in the reaction mixture (85). For complete lysis an appropriate balance of EDTA, lysozyme, and "Tris" was required. The optimum concentrations of lysozyme and EDTA varied from species to species and ranged from 10 to 17 and 130 to 530 μg./ml., respectively. The cells used were grown in a liquid medium for 18 to 24 hr. at 30°C., washed twice in distilled water and suspended in a concentration such that an optical density of 0.6 to 0.7 at 660 mμ. (1 cm. cuvettes) in the complete reaction mixture was obtained. Lysis was complete after 5 min. at room temperature.

In a following paper (71) Repaske points out that the growth medium, the physiological age of the cells, and the interval between harvesting and

lysing influence the degree of lysis. The order of addition of each reagent was also found to be of importance. The formation of protoplast-like bodies of *A. vinelandii* was also mentioned by Repaske. They were prepared in 0.3 *M NaCl* but had to be washed and resuspended in 0.1 *M* phosphate, pH 7, for stabilization.

Mahler & Fraser (54, 55) adopted Repaske's lysis method in their studies on phage reproduction in bacterial "protoplasts." *E. coli* cells, 1.1 $\times$ $10^9$ or less in the exponential growth phase, were suspended in 10 ml. of a mixture of "Tris" buffer, pH 8.0, and sucrose (final concentrations 0.033 *M* and 0.5 *M*, respectively). To this suspension, 0.1 ml. of 0.1 per cent lysozyme and, 2 min. afterwards, 0.05 ml. of 4 per cent EDTA were added. The experiments were conducted at 0°C. or at room temperature. The formation of "protoplasts" was completed within 10 to 30 min. The lysozyme-EDTA method for lysing *E. coli* cells was used also by Rickenberg (56) who introduced some modifications of the reagent concentrations.

It seems to the present author that a successful preparation of protoplast-like bodies of Gram-negative bacteria according to the methods just described, requires a rather precise adjustment of the experimental conditions. Thus, factors such as the age of the bacteria, the handling of the cells, the concentrations of the reagents, and the order in which the reagents are added to the reaction mixture have to be carefully controlled.

*The use of enzymes other than lysozyme. Autolytic procedures.*—Many more or less well-defined enzymes or enzyme systems, other than egg white lysozyme, have been reported to cause lysis of bacterial cells or cell walls. Some of these lytic agents [reviewed by Welsch (86); Mitchell & Moyle (52); Nomura & Hosoda (87); and Salton (74)] have been used for the preparation of protoplasts or protoplast-like bodies of various bacterial species.

Amano *et al.* used an extract of leucocytes for the conversion of *E. coli* cells into spherical, osmotically fragile bodies (57). The bacteria were grown on a solid medium for 18 hr. They were then harvested and suspended in 0.03 *M* phosphate of pH 7. The bacterial suspension was adjusted to a reading of $D_{550} = 0.03$ on the Coleman junior spectrophotometer and 0.3 ml. of it was mixed with 0.2 ml. of 1.3 *M* sucrose. A 0.5 ml. amount of extract, containing material from 1.2 $\times$ $10^8$ leucocytes, was then added. Protoplast-like bodies appeared after 2 hr. at 37°C. The process was accelerated by the addition of lysozyme.

The enzymatic extract used by Amano *et al.* seems to be related to the "phagocytin" described by Hirsch (88).

Panijel & Huppert used an extract of bacteria previously infected by phages as an agent for the preparation of "protoplasts" (58). *E. coli* cells were harvested in the exponential growth phase, centrifuged, and resuspended in 0.1 *M* "Tris" buffer of pH 8 at a concentration of 2 to 4 $\times$ $10^9$ cells/ml. An 0.8 ml. quantity of bacterial suspension was mixed with 1 ml. of 1 *M* sucrose and 0.2 ml. of distilled water. To this mixture, 0.05 to 0.3 ml.

phage lysate was added and then, after an additional 2 min., 0.03 ml. of 4 per cent EDTA. The conversion of cells into "protoplasts" was completed in 10 min. at 37°C.

"Protoplasts" of *B. cereus* and *B. megaterium* cells were prepared by Dark & Strange (48), using an extract from spores or sporulating cells of the same organisms. Bacteria in the stationary growth phase were harvested and resuspended in buffer of pH 7. Then, 0.2 ml. bacterial suspension, containing about 10 mg. bacterial dry weight/ml., 50 mg. sucrose and 50 μg. $Co^{2+}$ (enzyme activator) were added to 0.5 ml. of enzyme solution. After 20 to 60 min. at 37°C., the formation of spherical bodies was completed.

The ejection of protoplasts from *B. anthracis* cells as described by Stähelin (34, 51) was effected by suspending cells in Ringer's solution (0.9 per cent NaCl, 0.03 per cent KCl, and 0.026 per cent anhydrous $CaCl_2$). In this medium, 80 to 95 per cent of the cells were converted into protoplasts within a few hours at room temperature. Stähelin obtained the highest proportion of converted cells when using bacteria that had grown for 20 hr. at 37°C. in a medium rather similar to that described by Gladstone & Fildes (89). Only 2 out of 20 strains of *B. anthracis* yielded protoplasts using this procedure.

Mitchell & Moyle converted cells of *S. aureus* into "protoplasts" by incubating cells at 25°C. in 1.2 *M* sucrose containing 0.2 *M* NaCl, 0.01 *M* acetate buffer of pH 5.8 and 0.01 *M* iodoacetate (final concentrations). Rapid growth of the bacteria at harvesting was a prerequisite for the development of osmotic fragility.

The same workers prepared "protoplasts" of *E. coli* by incubating cells at 25°C. in 0.3 *M* sodium malonate (pH 7) containing 0.5 *M* L-arabinose. The rate of "protoplast" formation was greater the faster the growth of the bacteria at harvesting. Half of the cells harvested in the exponential growth phase became osmotically fragile in less than 60 min. at 25°C.

*Procedures using metabolic disturbances.*—In healthily growing bacteria the amounts of the various constituents of each cell increase in a balanced manner leading to the division of the cell. If, however, the synthesis of some essential cell constituent is inhibited, normal growth is rendered impossible and may be replaced by an unbalanced form of growth. Several cases of such abnormal growth, caused by metabolic deficiencies or drug action and often leading rapidly to the death of the organisms, are known [for a review see McQuillen (70)]. If the synthesis of some of the cell wall constituents is selectively inhibited, one could expect the formation of bacteria deprived more or less completely of their walls, i.e., the formation only of protoplasts or protoplast-like bodies (provided that a stabilizing agent is present in the medium). Several investigators have demonstrated the possibility of such conversions.

Meadow, Hoare & Work (68), Davis & Bauman (69), and McQuillen (70) obtained a conversion of *E. coli* cells into spherical, osmotically fragile bodies by growing a mutant (90), blocked in the synthesis of DAP in a

medium 0.6 *M* with respect to sucrose and containing no DAP (70). Formation of "protoplasts" followed after 2 to 3 hr. growth at 34°C. (68). The conversion is understandable when one takes into account the fact that DAP is mainly found in the cell wall fraction of bacteria (32).

Liebermeister & Kellenberger (59), Lederberg (60, 61), and Hahn & Ciak (62) showed that the metabolic disturbances caused by penicillin in sensitive bacteria can result in the conversion of the cells into osmotically fragile spheres. The "protoplasts" obtained by Liebermeister & Kellenberger were, however, relatively unstable since no stabilizing agent was present in the medium. Lederberg used 0.6 *M* sucrose as stabilizer. Overnight cultures of *E. coli,* strain K12, were diluted fourfold with growth medium supplemented with sucrose (5 to 20 per cent), penicillin (100 to 1000 units/ml.) and $MgSO_4$ (0.1 to 0.2 per cent). Within 2 to 3 hr. the cells were quantitatively converted into spheres.

Lederberg's penicillin technique has been adopted by Chargaff, Schulman & Shapiro (66) and by McQuillen (70).

### Biochemical and Biological Capabilities of Protoplasts and Protoplast-like Bodies

Numerous investigations on protoplasts have made it evident that the removal of the cell wall does not affect to any large extent most of the biochemical and biological activities of the bacterial cell. Weibull showed that *B. megaterium* cells and protoplasts, respectively, have the same endogenous respiration and oxidize glucose at an identical rate (21). Various nutrilites are incorporated into the protein and nucleic acid fractions of protoplasts [McQuillen (80); Oparin, Gelman & Zhukova (36, 91); Bridoux & Hanotier (37a); Oparin *et al.* (92); and Hunter *et al.* (78)]. The rates of incorporation are comparable with those characteristic for intact cells (80). The formation of adaptive enzymes by protoplasts was demonstrated by Wiame, Storck & Vanderwinkel (41), Landman & Spiegelman (77), and McQuillen (31, 93). Britt & Gerhardt (38) and Britt (39) found that protoplasts of *M. lysodeikticus,* like intact cells of this organism, are able to form an internal pool of lysine. Salton & McQuillen, (81), Brenner & Stent (82), and Mutsaars (42) showed that phage multiplication can take place in protoplasts. Salton demonstrated that cells of *B. megaterium* committed to spore formation [Hardwick & Foster (94)] could form protoplasts that were able to develop spores (95). McQuillen noted an increase of several hundred per cent in the dry weight and the size of protoplasts of *B. megaterium* grown in certain media (76). Some of the growing protoplasts developed dumb-bell shaped bodies, indicating an incipient division process. Completed divisions were, however, not observed.

Briefly, almost all of the activities exhibited by the bacterial protoplasts in intact cells can be observed in cell wall-free protoplasts as well. The only exceptions seem to be locomotion, the complete sequence of events leading to spore formation, complete divisions, and synthesis of a cell wall

structure. To the author's knowledge no reports of such activities have appeared so far.

In the case of osmotically fragile bacterial bodies ("protoplasts") that may still retain some of the cell wall structure, it is of interest to note that growth, resynthesis of a rigid wall and reappearance of rod-shaped bacteria have all been observed by Lederberg (60), Jeynes (73), and Zinder & Arndt (53). Furthermore, such forms may, in contrast to true protoplasts [Weibull (21)], absorb phage particles [Mahler & Fraser (54); Fraser & Mahler (55); and Spizizen (63)].

## Permeability and Osmotic Function of Protoplasts and Protoplast-like Bodies

Numerous studies have been performed on the permeability of whole bacterial cells [for a review see Mitchell & Moyle (96)]. These studies, performed with various experimental techniques, strongly favor the view that the exchange of matter between the interior of the cell and the external medium is regulated by a semipermeable membrane at the boundary of the protoplast, the so-called cytoplasmic membrane. The rigid cell wall, on the other hand, seems to be rather inert in this respect. Furthermore, it is probable that the low molecular weight compounds and the water present inside the protoplasts form a normal solution which exerts a considerable osmotic pressure against the cytoplasmic membrane.

Studies on protoplasts have given additional evidence for the presence of a permeability barrier in bacteria. One of the most characteristic properties of bacterial protoplasts, i.e., their osmotic fragility, is easily interpretable if an appreciable osmotic pressure exists inside the cell. In intact cells the mechanical strength of the cell wall prevents the disruption of cells suspended in dilute media or pure water. Once the cell wall is removed, however, this pressure easily disrupts the more fragile cytoplasmic membrane unless an osmotic counterpressure is applied. This opposing pressure can be obtained by adding to the medium a solute that does not penetrate into the protoplast, e.g., sucrose.

When protoplasts are lysed in dilute buffers they leave membranous "ghosts" as visible residues together with more or less numerous granular structures. The size and shape of the "ghosts" strongly suggest that they represent cytoplasmic membranes to a large extent free from other protoplasmic material [Tomcsik & Guex-Holzer (20); Weibull (21); Mitchell & Moyle (52); Weibull & Thorsson (33)]. Sometimes several spherical or irregularly formed "ghosts" may result from each protoplast but under appropriate conditions a 1 : 1 ratio is observed [Weibull (97)].

The permeability properties of protoplasts (protoplast-like bodies) of *M. lysodeikticus, S. lutea,* and *S. aureus* were investigated by Mitchell & Moyle (37, 52, 96). They prepared protoplasts in a medium containing sufficient sucrose to ensure stability. The protoplasts were then centrifuged, resuspended in 1.2 *M* NaCl and pipetted into 1.5 molal solutions of solutes

of which the permeability was to be measured. The degree of lysis and the rate of the lytic process was measured turbidimetrically. If no lysis was observed, the investigators concluded that the solute did not penetrate into the protoplasts and could therefore replace sucrose as a stabilizer. Lysis, on the other hand, would indicate a more or less rapid permeation of the protoplasts. In this way, Mitchell & Moyle found that metabolically inactive protoplasts of *M. lysodeikticus* and *S. lutea* were only slowly permeated by NaCl, KCl, $NH_4Cl$, $MgCl_2$, NaBr, KBr, $K_2SO_4$, sodium and potassium acetate (pH 9), phosphate, sodium glutamate, lysine hydrochloride, and various hexoses. The protoplasts rapidly lysed in glycerol, more slowly in erythritol and ribose solutions. "Protoplasts" of *S. aureus* resembled closely the protoplasts of *M. lysodeikticus* and *S. lutea* in their permeability properties. Mitchell & Moyle, moreover, showed that a close similarity exists between the permeation of the intact cells and the "protoplasts" of *S. aureus* by phosphate, chloride, and cyanate ions. In the experiments with intact cells, "the thick suspension technique" [Conway & Downey (98); Mitchell (99); Weibull (26)] was used to determine to what degree the various electrolytes penetrated the cells.

Mitchell & Moyle noted (96) that 50 per cent of the protoplasts (protoplast-like bodies) prepared from *M. lysodeikticus, S. leutea,* and *S. aureus* were stabilized in about 1 *M* NaCl or sucrose, i.e., solutions having an osmotic pressure of about 20 atm. They concluded that the last-mentioned figure represents the average osmotic pressure of the protoplasts.

The internal osmotic pressure of intact cells of *S. aureus* was determined by Mitchell & Moyle (96) by equilibrating a smear of cells against sucrose solutions of various osmolalities. The investigators showed that when the cell contained a normal amount of water, as determined by direct permeability measurements [Mitchell (99)], the decrease of vapor pressure caused by the intracellular solutes corresponded to that for about 1 *M* sucrose, i.e., to an osmotic pressure of 20 to 25 atm. Calculations based on the amounts of intracellular solutes and the amounts of protoplasmic water gave similar results. Thus, various measurements on intact cells and protoplasts (protoplast-like bodies) of *M. lysodeikticus, S. lutea,* and *S. aureus* indicate an internal osmotic pressure in the cells of about 20 to 25 atm.

If one assumes that the bacterial protoplasts are surrounded by a semipermeable membrane, a decrease in the volume of these bodies following an increase of the concentration of a solute in the medium and vice versa would indicate that they are less permeable to this solute than to water. In quantitative terms the volume changes should follow the law $P(V - b) =$ const., where $P$ represents the osmotic pressure of the external medium, $V$ the volume of the protoplast, and $b$ the volume of that part of the protoplast that does not respond to osmotic influences. Cells of higher organisms follow this law within certain limits [Lucké (100)].

Stähelin found (34) that protoplasts of *B. anthracis* [not of *Bacillus* M as stated in reference (50)] follow the law approximately when sus-

pended in sucrose or NaCl solutions of varying concentrations. This suggests that the protoplasts are impermeable to these solutes.

Weibull investigated the osmotic properties of protoplasts of *B. megaterium*, strain KM, and, like Stähelin, followed the volume changes of the protoplasts when they were suspended in solutions of various osmotic pressures (22). It was found that sucrose, glucose, and polyethylene glycol but not urethan caused shrinkage of the protoplasts. The volume changes followed the law $P(V - b) = \text{const}$. The quantity $b$ in this equation was found to be equivalent to about 35 per cent of the protoplast volume of the intact cell. In concentrated phosphate buffers, a shrinkage of the protoplasts was noted but at the same time many of these bodies disintegrated.

Using the thick suspension technique, Weibull showed that the protoplasts of intact *B. megaterium* cells under semianaerobic conditions are impermeable to sucrose, glucose, and phosphate but permeable to urethan (26). This suggests that the permeability properties of *B. megaterium* protoplasts are about the same, irrespective of whether or not the protoplast is surrounded by the cell wall.

From the evidence quoted above, it seems that the removal of the cell wall from bacterial cells does not affect the permeability properties of the protoplasts. Other data suggest, however, that some differences in this respect do exist between the protoplasts of the intact cells and free protoplasts. Thus, Spiegelman found (79, 101, 102) that the DNA and RNA of *B. megaterium* protoplasts could be degraded by deoxyribonuclease and ribonuclease whereas intact cells were not affected by these enzymes. Groth, on the other hand, found (103) that ribonuclease markedly inhibited growth and protein synthesis of intact *B. megaterium* cells. Much of the degraded nucleic acids remained in the protoplasts. Spiegelman's findings must, in all probability, imply that these enzymes can penetrate into the protoplasts. Perhaps the phenomena described by Spiegelman could be explained by considering some of Weibull's findings concerning the osmotic properties of *B. megaterium* protoplasts (22). The latter investigator found that the osmotic pressure of the medium before the cell wall is removed has little or no influence on the total volume of the protoplasts. Once the protoplasts are formed, on the other hand, they respond to changes in the osmotic pressure of the medium; cf. the preceding paragraphs. Perhaps these findings could be explained by assuming a transient increase in permeability of the cytoplasmic membrane at the moment of removal of the cell wall, permitting some substances to enter the protoplast. It is noteworthy in this connection that Spiegelman found a decreasing effectiveness of various enzymes toward bacterial protoplasts if the enzymes were added after the protoplasts had been formed.

In this connection it might perhaps be worth emphasizing that the stability of free bacterial protoplasts is affected by several other factors besides the osmotic pressure of the medium. Protoplasts thus lyse rapidly when they come into contact with oxygen (21) but are stabilized by

Mg ions (97). According to Mitchell & Moyle, "protoplasts" of *S. aureus* lyse when cooled to 2°C. (96). Sistrom recently found (104) that "protoplasts" of an *E. coli* strain, which accumulated lactose without hydrolyzing this compound, lysed in a medium containing lactose. Sistrom concluded that the lysis was caused by an increase of the internal osmotic pressure of the "protoplasts."

### Degraded Protoplasts. Subunits of These Bodies

Bacterial protoplasts and related forms are fragile structures that can be degraded in various ways, e.g., by shocking them osmotically, by shaking them with air, or by enzymic treatments. From the resultant mixture of degradation products, elements of various kinds can be isolated. In this way protoplasts and protoplast-like bodies may serve as a starting point for further studies on structural subunits of the bacterial cell.

Oparin, Gelman & Zhukova (36, 91) and Oparin, Gelman & Deborin (92) studied the biochemical capabilities of *M. lysodeikticus* protoplasts prepared in sucrose solutions of various concentrations. They found that incorporation of labelled glycine ceased rather abruptly when the sucrose concentration was lowered below 0.44 *M*. Respiration was still more sensitive to a decrease in the osmotic pressure of the medium, being markedly inhibited at sucrose concentrations less than 0.64 *M*. The net increase of protein nitrogen in protoplasts incubated in a medium containing 19 amino acids proceeded analogously to the incorporation of labeled glycine in the absence of inhibitors. It was noted, however, that the incorporation of radioactive glycine was inhibited by azide, dinitrophenol, and biomycine whereas these inhibitors had no effect on the net protein synthesis in a medium containing a complete set of amino acids.

Incorporation of inorganic-P into organic-P fractions was found to be rather independent of the sucrose concentration of the medium, taking place at about the same rate in both 0.42 *M* and 0.84 *M* sucrose (91).

Oparin, Gelman & Zhukova included in their paper (36) electron micrographs of protoplasts prepared in sucrose solutions of various concentrations. These photographs indicate that decreasing the sucrose concentration causes a gradual degradation of the structural integrity of the protoplasts.

Several investigations have shown that the synthetic capabilities of *B. megaterium* protoplasts (e.g., incorporation of labelled nutrilites, formation of spores and adaptive enzymes, support of phage multiplication) are destroyed when these bodies are lysed osmotically [McQuillen (31, 80, 93); Bridoux & Hanotier (37a); Salton (95); and Salton & McQuillen (81)]. McQuillen found, however, that if lysis was performed by shaking the protoplast suspension with air instead of by lowering the concentration of the stabilizing agent, a preparation was obtained in which synthesis of amino acids and incorporation of amino acids into the protoplast protein took place (31).

Spiegelman and his associates have initiated extensive investigations on

the synthetic activities of protoplasts and protoplast-like bodies after treating them with various degrading agents [Landman & Spiegelman (77); Spiegelman (79, 101, 102); Ezekiel & Spiegelman (105); Aronson & Spiegelman (106); Ben-Porat & Spiegelman (64); and Nisman & Spiegelman (65)]. The account given below will be restricted to data pertaining directly to the nature of bacterial protoplasts.

Landman & Spiegelman (77) and Spiegelman, Li & Landman (107) studied the influence of trypsin, lipase, ribonuclease, and deoxyribonuclease on the chemical composition of intact cells and protoplasts of *B. megaterium* and the effect of these agents on the synthesis of an adaptive enzyme, β-galactosidase, in cells and protoplasts. It appeared that not one of the enzymes studied attacked whole cells whereas striking modifications were obtained with protoplasts. Thus, a complete lysis resulted when lipase or trypsin was added to a suspension of protoplasts. A partial degradation was effected by ribonuclease or deoxyribonuclease. These enzymes selectively degraded the RNA and DNA of protoplasts suspended in phosphate into acid soluble fragments. [Brenner, on the other hand, noted a complete lysis by ribonuclease of *B. megaterium* protoplasts suspended in sucrose (83)]. The enzyme-forming capacity of the protoplasts was drastically inhibited if more than 30 per cent of the RNA was broken down. On the other hand, up to 99 per cent of the DNA could be degraded without affecting the formation of β-galactosidase very much. About 60 per cent of the degraded DNA remained inside the protoplasts.

Spiegelman, Wolin & Liu (108) investigated the effect on the structure and activities of *B. megaterium* protoplasts which resulted from controlled changes in the osmotic pressure of the medium. Succinate (0.5 *M*) was used as the stabilizing agent. After preparation of the protoplasts the osmotic pressure of the medium was suddenly lowered by the addition of water. The osmotically shocked protoplasts were centrifuged and the resulting sediment resuspended in 0.5 *M* succinate.

It appeared that the RNA and protein were most easily removed from the protoplasts by osmotic shocking, DNA being more resistant to this treatment. The enzyme-forming capacity of the preparations remained unimpaired or was even enhanced, until the RNA/protein ratio was very much reduced. This happened when the suspension was diluted more than fourfold, which effected a proportionally greater solubilization of RNA than of protein.

Microscopically, the osmotically shocked protoplasts retained the shape and size of the untreated bodies but had a lower optical density. In appropriate media they were able to synthesize DNA, RNA, and protein. In contrast to intact protoplasts, however, the osmotically shocked preparations displayed a synthetic activity which was highly unbalanced. Furthermore, enzymes and strong salt solutions inhibited much more readily the synthetic activities of osmotically shocked protoplasts than those of intact ones.

Investigations on osmotically shocked "protoplasts" of *E. coli*, prepared

by penicillin treatment, were recently reported by Ben-Porat & Spiegelman (64) and Nisman & Spiegelman (65). The former workers studied interactions between shocked "protoplasts" and disrupted $T_2$ phage particles. Nisman & Spiegelman (65) investigated amino acid incorporation into "protoplasts." These bodies were shocked by lowering the concentration of the stabilizing agent, sucrose, from 18 or 10 per cent to about two per cent. Net protein synthesis in the degraded "protoplasts" occurred only in a medium that contained, among other substances, a complete set of amino acids.

The structures found in osmotic lysates of bacterial protoplasts have been studied by various investigators. The morphological characteristics of the "ghosts" resulting from lysed protoplasts were mentioned in the section in this review dealing with permeability problems. The cytoplasmic membrane of the bacterial cell evidently represents a major component of the "ghosts." However, the "ghosts" may contain additional cytoplasmic material, often in the form of granules [Weibull (109); Georgi, Militzer & Decker (110); and Weibull & Thorsson (33)]. The nature of the granular material seems to vary from one bacterial species or strain to another (33, 109, 110).

According to Mitchell & Moyle (96) the cytoplasmic membrane of *S. aureus,* isolated from "protoplasts" of this organism, contains 41 per cent protein and 22.5 per cent lipide. Weibull found 13 to 21 per cent lipide in the "ghosts" of *B. megaterium,* strain M (84). According to Vennes (30), "ghosts" of *B. megaterium,* strain KM, contain 11 per cent RNA. Georgi, Militzer & Decker reported the presence of about 10 per cent lipide in the "red fraction" of *Bacillus stearothermophilus* (110). This fraction, obtained by lysozyme lysis of the organism studied, probably represents a "ghost" fraction. McQuillen reported (70) that the cytoplasmic membrane of *M. lysodeikticus* contains 50 per cent protein, 28 per cent lipide, and 18.5 per cent carbohydrate (16 per cent mannose and 2.5 per cent hexosamine). No mannose and 16 per cent hexosamine were found in the cell wall of the same organism.

Weibull noted that the cytochrome system of *B. megaterium,* strain KM, sedimented with the "ghost" fraction in differential centrifugations (109). Georgi, Militzer & Decker found that the cytochrome-*c* and adenosinetriphosphatase were located in the "red fraction" of *B. stearothermophilus* cells (110).

According to Mitchell & Moyle (111), the cytoplasmic membrane of *S. aureus* contains at least 90 per cent of the cytochrome system of the cells, together with the bulk of the following enzymes: lactic, malic and formic dehydrogenase, "malic" enzyme, and acid phosphatase. Glucose-6-phosphate dehydrogenase and glucose-6-phosphatase, on the other hand, were found in the soluble part of the protoplasm.

Storck & Wachsman (112) performed a detailed investigation on the localization of the respiratory enzymes of *B. megaterium,* strain KM. Pro-

toplasts of this organism were lysed osmotically and fractionated into "ghosts" and soluble protoplasm. Determinations based on dye reduction showed that the enzyme systems responsible for all the succinate, DL-lactate, and α-ketoglutarate oxidation were located in the "ghost" fraction. The soluble part of the protoplasm accounted for all the glucose, pyruvate, citrate, and *cis*-aconitate oxidation of the total lysate. Oxidation of L-malate was effected both by the "ghosts" and the soluble protoplasm.

Several investigations have been performed on particulate, enzymatically active fractions isolated from mechanically disintegrated bacterial cells [for a review see Alexander (113)]. A comparison between the data gained from these investigations and from studies on lysed protoplasts and "ghosts" strongly suggests that, in many cases at least, the material isolated from the mechanically treated bacteria represents disintegrated cytoplasmic membranes.

Vennes (30) and Vennes & Gerhardt (29, 114) investigated the immunological properties of the following structural elements isolated from *B. megaterium* cells: spores, spore coats, flagella, capsular material, cell walls, protoplasts, cytoplasmic membranes, intracellular particles, and nuclear bodies. The four last-mentioned structures were isolated from lysozyme-treated bacteria. The soluble part of the cytoplasm was, however, not included in the investigations. Data gained from agglutination and complement-fixation tests showed that an antiserum prepared against one of the isolated structures did not react significantly with any of the others. Thus, each of the structures appeared to be antigenically highly distinct.

### Relationships Between Bacterial Protoplasts (Protoplast-like bodies) and Bacterial L-Forms

Several workers have suggested that the bacterial L-forms may be protoplasmic in their nature [Liebermeister & Kellenberger (59); Klieneberger-Nobel (115); Weibull (50); and Lederberg (61)]. These forms [reviewed by Klieneberger-Nobel (116); Dienes & Weinberger (117); Tulasne (118, 119); and Schellenberg (120)] are highly pleomorphic: they may consist of granular, vesicular, or branched structures. The L-forms, moreover, are characterized by plasticity and by sensitivity towards osmotic changes in the medium. These facts indicate that no rigid envelope surrounds the forms. It could be asked, however, whether the rigid wall of normal cells is lost completely or merely modified to some extent as a consequence of the conversion of rod-shaped bacteria into pleomorphic L-forms. If the L-forms are completely free of cell walls they could be regarded as protoplasts endowed with the capacity for reproduction; if not, they should rather be compared with the bacterial structures which have been designated as protoplast-like bodies in the terminology section of this review.

L-forms may be produced when bacteria are subjected to an unusual or unfavorable environment. Thus, sublethal doses of penicillin are often used

for the conversion of normal bacteria into L-forms [cf. the preparation of "protoplasts" (60, 62)]. The first stages of this conversion, as studied by optical methods, have been described in different ways. In the case of *Proteus*, Freundt (121), Tulasne (119), Schellenberg (120), and Bartmann & Höpken (122) found that the protoplasm was extruded from the bacteria, leaving the cell walls as empty filaments (plasmoptysis). Other investigators [Stempen (123) and Taubeneck (124)] described the process more as a deformation of the whole cells and found no evidence for plasmoptysis. Dienes reported the occurrence of plasmoptysis (125) as well as a general swelling of the bacterial body (117, 126). Klieneberger-Nobel found that during the first stages of the conversion process filamentous bacterial cells broke up into small granules which later gave rise to L colonies (127).

Some electron microscope investigations on L-forms have been performed but to the author's knowledge only on unsectioned material so far [Smith, Mudd & Hillier (128); Bringmann (128a); Dienes (129); Kandler, Kandler & Huber (130); Klieneberger-Nobel (131); Rubio Huertos & Desjardins (132); and Pease (133, 134)]. Any definite conclusions as to the presence or absence of a (modified) cell wall can hardly be drawn from these studies.

Dienes, Weinberger & Madoff (135) found serological relationships between the normal and L-forms of two *Proteus* strains whereas no such common antigens were detected in a third strain. Poetschke, Killisch & Uehleke (136) found cross-reactions in similar experiments carried out on *Proteus morganii*. Tulasne reported that O antigens but no H antigens were present in the L-form of *Proteus vulgaris* (118).

Sharp, Hijmans & Dienes (137) studied the chemical and immunological properties of group A streptococci and the corresponding L-form. They found that a group-specific polysaccharide, known to be a major component of the cell wall of the normal bacteria, was lacking in the L-forms. The investigators concluded that the L-form studied closely resembles protoplasts prepared from other bacteria.

Kandler & Zehender (138) analyzed several strains of *P. vulgaris* for DAP. They were unable to demonstrate the presence of this compound in stable L-forms but found it in unstable ones (i.e., such strains that reverted sooner or later to the normal bacillary form). Just as Work (32) regarded DAP as a characteristic component of the cell wall, so Kandler & Zehender concluded from their results that the synthesis of cell wall material is blocked in the stable L-forms.

Kandler prepared his analysis material by washing the L-bodies with water. Weibull (139) precipitated in a similar investigation the L-bodies directly in the DAP-free medium using cold trichloroacetic acid, and noted the presence of DAP in the precipitated material. Considerably greater amounts of this compound were, however, found in similarly treated normal cells. Weibull thus reached a conclusion somewhat different from Kandler's,

i.e., that stable L-forms are mainly, but not entirely, protoplasmic in their nature.

The somewhat contradictory results quoted above concerning the more or less complete removal of the normal cell wall following the formation of bacterial L-forms could, in the case of the chemical investigations, be explained by the different methods used for isolating the material to be analyzed. The discrepancies between the various morphological (and immunological) studies may well result from inherent structural differences of the investigated L-forms. Thus, Dienes (126) described two types of L colonies (3A and 3B) derived from the same bacterial culture. Perhaps some L-forms are completely devoid of the cell wall structure of the corresponding bacillary forms, whereas others (stable or unstable) retain part of this structure.

### Genetic Studies on "Protoplasts"

The permeability properties of the cytoplasmic membrane of intact bacteria and of free protoplasts are, in all probability, very much the same. Some data suggest, however, that the dissolution of the cell wall may facilitate to some degree the entrance of certain substances into the bacterial protoplasts (see section of this review dealing with permeability problems). It could therefore be argued, as has been done by Lederberg (60) and Chargaff, Schulman & Shapiro (66), that protoplasts and related forms might be suitable tools in recombination experiments and other genetic investigations. Preliminary studies in this field, performed by Lederberg (60) are, however, not too promising in this respect. This investigator found that "protoplasts" of *E. coli* retained their ability to mate but no new types of genetic recombination were revealed.

Chargaff, Schulman & Shapiro (66) studied the occurrence of genetic transformation in "protoplasts" of *E. coli*. Purified DNA prepared from "protoplasts" of wild type *E. coli* was allowed to act on similar bodies obtained from a lysine-requiring mutant of the same strain. The "protoplasts" of the metabolically defective mutant were then made to revert to the normal, bacillary growth form and the percentage of prototrophic cells in the progeny was determined. A comparison between the results of such experiments and of controls carried out on cells which had gone through the "protoplast" stage but which had not been treated with DNA showed that this treatment produced up to a twentyfold increase in the number of prototrophic cells in the progeny of the "protoplasts."

The investigation just quoted evidently demonstrates, like Lederberg's studies (60), that genetic phenomena can take place in bacterial "protoplasts." Chargaff, Schulman & Shapiro did not, however, include in their report data concerning the effect of purified DNA on intact cells; additional evidence seems therefore desirable in order to ascertain whether or not the degradation of the cell wall facilitates the entrance of genetically active material into the protoplasmic part of bacterial cells.

## Mode of Action of Penicillin in the Light of Studies on Bacterial "Protoplasts"

Lederberg (61), Hahn & Ciak (62), Park & Strominger (140), and Weidel & Primosigh (140a) pointed out that conversion of rod-shaped bacto the normal growth form in penicillin-free media, the penicillin treatment in a sucrose medium, strongly suggests that this antibiotic inhibits the mechanism of cell wall formation. Since the "protoplasts" are capable of reverting to the normal growth form in penicillin-free media, the penicillin treatment does not seem to affect directly the structure and functions of the protoplasmic part of the bacteria to any large extent. Additional evidence supports this view. As a result of work by Park & Johnson (141) and Park (142), it appeared that penicillin causes an accumulation in whole *S. aureus* cells of a nucleotide consisting of uridine-5′-pyrophosphate linked to an *N*-acetylamino sugar and a peptide composed of D-glutamic acid, L-lysine, and DL-alanine in the ratio 1 : 1 : 3. Analyses of isolated cell walls of the same organism [Strominger, Park & Thompson (143)] revealed the presence of the same amino sugar, D-glutamic acid, L-lysine, and DL-alanine in amounts approaching the ratio 1 : 1 : 1 : 3, the ratio found in the above-mentioned nucleotide. Furthermore, a limited acid hydrolysis of the cell walls yielded fragments very similar to those derived by similar treatment of the nucleotide (144).

The data quoted above could readily be interpreted by assuming that penicillin selectively inhibits the polymerization of the cell wall building units, thus preventing the formation of a rigid, protective framework around the protoplasm. The general lysis of the cell, characteristic of penicillin treatment, would thus be a secondary phenomenon caused by the fragility of the cytoplasmic membrane.

According to several of the earlier theories dealing with the mode of action of penicillin [for a review see Cooper (145)] the primary effect of this antibiotic consists of a damaging of the osmotic barrier (cytoplasmic membrane) of the susceptible bacteria. A recent article advocating this view is that of Prestidge & Pardee (146). Among other things these workers found that inhibition of protein synthesis in *E. coli* cells prevents the damaging effects of penicillin. It was supposed that penicillin causes the formation of an enzyme which attacks the cytoplasmic membrane and thus initiates the destruction of the cell structure. Prestidge & Pardee reported, as supporting their view, the observation that a fraction of disintegrated *E. coli* cells, previously treated with penicillin, is able to lyse *B. megaterium* protoplasts. The same fraction of untreated cells was not effective in this respect. Whole extracts of such cells, on the other hand, could lyse protoplasts but usually less efficiently than similar preparations from penicillin-treated cells. The lytic agent in extracts from penicillin-treated cells was, moreover, more slowly destroyed by heat than the agent in untreated cells.

In the present author's opinion the evidence presented so far favors the view that penicillin causes primarily the inhibition of cell wall formation, the breakdown of the osmotic barrier (cytoplasmic membrane) being a secondary effect.

### Protoplasts of Yeasts

Like bacteria, yeast cells are provided with a rigid cell wall that is not easily degraded mechanically or by other means. Recently, however, two reports have appeared [Nečas (147) and Eddy & Williamson (148)] showing the possibility of separating the yeast protoplast from the cell wall without affecting severely the structure and functions of the protoplast.

Nečas mentioned two methods for the isolation of protoplasts of *Saccharomyces cerevisiae:* mechanical pressure on the yeast cells and partial autolysis of a yeast culture. Several plasma droplets resulted from each yeast cell but according to Nečas one of them contained the whole nucleus.

The plasma droplets containing nuclei gave evidence of real growth. Some of these plasma formations eventually degenerated but others developed further. Thus, sometimes the plasma contracted, the vacuoles disappeared, and dense spherical bodies appeared. A membrane appeared on the surface of these bodies, which contained several nuclei, and a budding process started. After a few generations, cells appeared which, in most cases, resembled the original yeast cells morphologically. Deviations in this respect and in the physiological properties were, however, encountered.

Eddy & Williamson (148) used an enzymatic preparation from the snail *Helix pomatia* for dissolving the cell wall of *S. cerevisiae* and *Saccharomyces carlsbergiensis.* The dissolution was conducted in a medium containing 0.55 *M* rhamnose (glucose caused lysis of the protoplasts) and 0.005 *M* citrate-phosphate buffer, pH 5.8 (final concentrations). After 18 hr. at room temperature, more than 90 per cent of the yeast cells were converted into protoplasts. Young cells had to be used in order to get complete digestion of the cell wall. The release of the protoplasts evidently proceeded as a kind of plasmoptysis, the protoplast extruding through a hole in the cell wall, which developed during the enzymatic treatment. Eventually the cell wall dissolved completely, leaving a suspension of protoplasts essentially free from whole cells and debris. Eddy & Williamson did not report anything about the physiological activities of the isolated protoplasts.

## LITERATURE CITED

1. Robinow, C. F., *J. Hyg.*, **43**, 413–23 (1944)
2. Robinow, C. F., in Dubos, R. J., *The Bacterial Cell*, 355–77 (Harvard University Press, Cambridge, Mass., 377 pp., 1945)
3. Houwink, A. L., and van Iterson, W., *Biochim. et Biophys. Acta*, **5**, 10 (1950)
4. van Iterson, W., *Proc. Intern. Conf. Electron Microscopy, London, 1954, 3rd Conf.*, 602–7 (Royal Microscopical Soc., London, England, 705 pp., 1956)

5. Chapman, G. B., and Hillier, J., *J. Bacteriol.*, **66**, 362–73 (1953)
6. Weibull, C., *Acta Chem. Scand.*, **4**, 268–76 (1950)
7. Salton, M. R. J., *Biochim. et Biophys. Acta*, **10**, 512 (1953)
8. Schachman, H. K., Pardee, A. B., and Stanier, R. Y., *Arch. Biochem. Biophys.*, **38**, 245–60 (1952)
9. Fischer, A., *Vorlesungen ueber Bakterien*, 20–31 (Verlag von Gustav Fischer, Jena, Germany, 374 pp., 1903)
10. Ellis, D., *Zentr. Bakteriol. Parasitenk. I. Orig.*, **33**, 81–96 (1903)
11. Swellengrebel, N. H., *Ann. inst. Pasteur*, **21**, 448–65 (1907)
12. Garbowski, L., *Arch. Protistenk.*, **9**, 53 (1907)
13. Vahle, C., *Zentr. Bakteriol. Parasitenk. II*, **25**, 178 (1910)
14. Raichel, B., *Arch. Protistenk.*, **63**, 333 (1928)
15. Knaysi, G., *J. Bacteriol.*, **19**, 113–15 (1930)
16. Elo, J. E., *Ann. botan. soc. Zool-botan. Fennicae Vanamo*, **8**, 1–25 (1937)
17. Salton, M. R. J., *Symposium Soc. Gen. Microbiol.*, **6**, 81–110 (1956)
18. Knaysi, G., *Elements of Bacterial Cytology*, 2nd ed., 159–62 (Constock Publishing Co., Inc., Ithaca, N.Y., 375 pp., 1951)
19. Salton, M. R. J., *Nature*, **170**, 746–47 (1952)
20. Tomcsik, J., and Guex-Holzer, S., *Schweiz. Z. allgem. Pathol. u. Bakteriol.*, **15**, 517–25 (1952)
21. Weibull, C., *J. Bacteriol.*, **66**, 688–95 (1953)
22. Weibull, C., *Exptl. Cell. Research*, **9**, 294–304 (1955)
23. Cooper, P. D., Rowley, D., and Dawson, L. M., *Nature*, **164**, 842–43 (1949)
24. Salton, M. R. J., *J. Gen. Microbiol.*, **11**, ix (1954)
25. Welshimer, H. J., *J. Bacteriol.*, **66**, 112–17 (1953)
26. Weibull, C., *Exptl. Cell Research*, **9**, 139–47 (1955)
27. Salton, M. R. J., *J. Gen. Microbiol.*, **9**, 512–23 (1953)
28. Tomcsik, J., and Guex-Holzer, S., *Experientia*, **10**, 484–85 (1954)
29. Vennes, J. W., and Gerhardt, P., *Science*, **124**, 535–36 (1956)
30. Vennes, J. W., *A Serological Analysis of Isolated Cellular Structures in* Bacillus megaterium (Doctoral thesis, The University of Michigan, Ann Arbor, Mich., 1957)
31. McQuillen, K., *Symposium Soc. Gen. Microbiol.*, **6**, 127–49 (1956)
32. Work, E., *Nature*, **179**, 841–47 (1957)
33. Weibull, C., and Thorsson, K. G., in *Electron Microscopy*, 266–67 (Proceedings of the Stockholm Conference, September, 1956. Sjöstrand, F. S., and Rhodin, J., Eds., Almqvist & Wiksell, Stockholm, Sweden, 355 pp., 1957)
34. Stähelin, H., *Schweiz. Z. allgem. Pathol. u. Bakteriol.*, **17**, 296–310 (1954)
35. Colobert, L., *Compt. rend. soc. biol.*, **151**, 114–16 (1957)
36. Oparin, A. I., Gelman, N. S., and Zhukova, I. G., *Doklady Akad. Nauk S.S.S.R.*, **105**, 1036–38 (1955)
37. Mitchell, P., and Moyle, J., *J. Gen. Microbiol.*, **15**, 512–20 (1956)
37a. Bridoux, M., and Hanotier, J., *Biochim. et Biophys. Acta*, **22**, 103–10 (1956)
38. Britt, E. M., and Gerhardt, P., *Bacteriol Proc. (Soc. Am. Bacteriologists)*, 38 (1956)
39. Britt, E. M., *The Role of Surface Membranes in Bacterial Permeability* (Doctoral thesis, University of Michigan, Ann Arbor, Mich., 1956)

40. Few, A. V., Fraser, M. J., and Gilby, A. R., *Biochim. et Biophys. Acta*, **24**, 306–14 (1957)
41. Wiame, J. M., Storck, R., and Vanderwinkel, E., *Biochim. et Biophys. Acta*, **18**, 353–57 (1955)
42. Mutsaars, W., *Ann. inst. Pasteur*, **89**, 166–82 (1955)
43. Salton, M. R. J., in *Proc. Intern. Cong. Biochem., 3rd Congr., Brussels, 1955*, 404–10 (Liébecq, C., Ed., Academic Press, Inc., New York, N.Y., 544 pp., 1956)
44. Tomcsik, J., and Baumann-Grace, J. B., *Verhandl. naturforsch. Ges. Basel*, **67**, 218–38 (1956)
45. Baumann-Grace, J. B., and Tomcsik, J., *Experientia*, **13**, 148 (1957)
46. Strange, R. E., and Dark, F. A., *J. Gen. Microbiol.*, **16**, 236–49 (1957)
47. Strange, R. E., and Dark, F. A., *J. Gen. Microbiol.*, **17**, 525–37 (1957)
48. Dark, F. A., and Strange, R. E., *Nature*, **180**, 759–60 (1957)
49. Fischer, A., *Z. Hyg. Infektions-krankh.*, **35**, 1–58 (1900)
50. Weibull, C., *Symposium Soc. Gen. Microbiol.*, **6**, 111–26 (1956)
51. Stähelin, H., *Schweiz. Z. allgem. Pathol. u. Bakteriol.*, **16**, 892–901 (1953)
52. Mitchell, P., and Moyle, J., *J. Gen. Microbiol.*, **16**, 184–94 (1957)
53. Zinder, N. D., and Arndt, W. F., *Proc. Natl. Acad. Sci. U.S.*, **42**, 586–90 (1956)
54. Mahler, H. R., and Fraser, D., *Biochim. et Biophys. Acta*, **22**, 197–99 (1956)
55. Fraser, D., and Mahler, H. R., *Arch. Biochem. Biophys.*, **69**, 166–77 (1957)
56. Rickenberg, H. V., *Biochim. et Biophys. Acta*, **25**, 206–7 (1957)
57. Amano, T., Seki, Y., Fujikawa, K., Kashiba, S., Morioka, T., and Ichikawa, S., *Med. J. Osaka Univ.*, **7**, 245–56 (1956)
58. Panijel, J., and Huppert, J., *Compt. rend.*, **245**, 240–43 (1957)
59. Liebermeister, K., and Kellenberger, E., *Z. Naturforsch.*, **11b**, 200–6 (1956)
60. Lederberg, J., *Proc. Natl. Acad. Sci. U.S.*, **42**, 574–77 (1956)
61. Lederberg, J., *J. Bacteriol.*, **73**, 144 (1957)
62. Hahn, F. E., and Ciak, J., *Science*, **125**, 119–20 (1957)
63. Spizizen, J., *Proc. Natl. Acad. Sci. U.S.*, **43**, 694–701 (1957)
64. Ben-Porat, T., and Spiegelman, S., *Bacteriol. Proc. (Soc. Am. Bacteriologists)*, 136 (1957)
65. Nisman, B., and Spiegelman, S., *Bacteriol. Proc. (Soc. Am. Bacteriologists)*, 137 (1957)
66. Chargaff, E., Schulman, H. M., and Shapiro, H. S., *Nature*, **180**, 851–52 (1957)
67. Mitchell, P., and Moyle, J., *Nature*, **178**, 993 (1956)
68. Meadow, P., Hoare, D. S., and Work, E., *Biochem. J. (London)*, **66**, 270–82 (1957)
69. Davis, B. D., and Bauman, N., in Bauman, N., and Davis, B. D., *Science*, **126**, 170 (1957)
70. McQuillen, K., *J. Gen. Microbiol.*, **18**, 498–512 (1958)
71. Repaske, R., *Bacteriol. Proc. (Soc. Am. Bacteriologists)*, 130–31 (1957)
72. Colobert, L., *Compt. rend soc. biol.* (In press)
73. Jeynes, M. H., *Nature*, **180**, 867 (1957)
74. Salton, M. R. J., *Bacteriol. Revs.*, **21**, 82–99 (1957)
75. Tomcsik, J., *Proc. Soc. Exptl. Biol. Med.*, **89**, 459–63 (1955)
76. McQuillen, K., *Biochim. et Biophys. Acta*, **18**, 458–61 (1955)

77. Landman, O. E., and Spiegelman, S., *Proc. Natl. Acad. Sci. U.S.*, **41**, 698–704 (1955)
78. Hunter, G. D., Crathorn, A. R., and Butler, J. A. V., *Nature*, **180**, 383–84 (1957)
79. Spiegelman, S., *Nucleic Acids and the Synthesis of Proteins*, 232–67 (A symposium on the Chemical Basis of Heredity, McElroy, W. D., and Glass, B., Eds., The Johns Hopkins Press, Baltimore, Md., 848 pp., 1957)
80. McQuillen, K., *Biochim. et Biophys. Acta*, **17**, 382–90 (1955)
81. Salton, M. R. J., and McQuillen, K., *Biochim. et Biophys. Acta*, **17**, 465–72 (1955)
82. Brenner, S., and Stent, G. S., *Biochim. et Biophys. Acta*, **17**, 473–75 (1955)
83. Brenner, S., *Biochim. et Biophys. Acta*, **18**, 531–34 (1955)
84. Weibull, C., *Acta Chem. Scand.*, **11**, 881–92 (1957)
85. Repaske, R., *Biochim. et Biophys. Acta*, **22**, 189–91 (1956)
86. Welsch, M., *Rev. belge pathol. et med. exptl.*, **18**, Suppl. II (1947)
87. Nomura, M., and Hosoda, J., *J. Bacteriol.*, **72**, 573–81 (1956)
88. Hirsch, J. G., *J. Exptl. Med.*, **103**, 589–611 (1956)
89. Gladstone, G. P., and Fildes, P., *Brit. J. Exptl. Pathol.*, **21**, 161–73 (1940)
90. Davis, B. D., *Nature*, **169**, 534–36 (1952)
91. Oparin, A. I., Gelman, N. S., and Zhukova, I. G., *Biokhimiya*, **22**, 399–403 (1957)
92. Oparin, A. I., Gelman, N. S., and Deborin, G. A., *Arch. Biochem.*, **69**, 582–91 (1957)
93. McQuillen, K., *J. Gen. Microbiol.*, **13**, iv (1955)
94. Hardwick, W. A., and Foster, J. W., *J. Gen. Physiol.*, **35**, 907–27 (1952)
95. Salton, M. R. J., *J. Gen. Microbiol.*, **13**, iv (1955)
96. Mitchell, P., and Moyle, J., *Symposium Soc. Gen. Microbiol.*, **6**, 150–80 (1956)
97. Weibull, C., *Exptl. Cell Research*, **10**, 214–21 (1956)
98. Conway, E. J., and Downey, M., *Biochem. J. (London)*, **47**, 347–55 (1950)
99. Mitchell, P., *J. Gen. Microbiol.*, **9**, 273–87 (1953)
100. Lucké, B., *Cold Spring Harbor Symposia Quant. Biol.*, **8**, 123–32 (1940)
101. Spiegelman, S., *Enzymes: Units of Biological Structure and Function*, 67–89 (Gaebler, O. H., Ed., Academic Press, Inc., New York, N.Y., 624 pp., 1956)
102. Spiegelman, S., *Protein Synthesis in Protoplasts*, 185–92 (Ciba Foundation Symposium on Ionizing Radiations and Cell Metabolism, Wolstenholme, G. E. W., and O'Connor, C. M., Eds., J. & A. Churchill, Ltd., London, 318 pp., 1956)
103. Groth, D. P., *Biochim. et Biophys. Acta*, **21**, 18–23 (1956)
104. Sistrom, W. R., cited in Cohen, G. N., and Monod, J., *Bacteriol. Revs.*, **21**, 169–94 (1957)
105. Ezekiel, D. H., and Spiegelman, S., *Bacteriol. Proc. (Soc. Am. Bacteriologists)*, 137–38 (1957)
106. Aronson, A. I., and Spiegelman, S., *Bacteriol. Proc. (Soc. Am. Bacteriologists)*, 138 (1957)
107. Spiegelman, S., Li, T. K., and Landman, O., in Spiegelman, S., *Enzymes: Units of Biological Structure and Function*, 67–89 (Academic Press, Inc., New York, N.Y., 624 pp., 1956)

108. Spiegelman, S., Wolin, E., and Liu, G. B., in Spiegelman, S., *Nucleic Acids and the Synthesis of Proteins,* 232–67 (A Symposium on the Chemical Basis of Heredity, McElroy, W. D., and Glass, B., Eds., The Johns Hopkins Press, Baltimore, Md., 848 pp., 1957)
109. Weibull, C., *J. Bacteriol.,* **66,** 696–702 (1953)
110. Georgi, C. E., Militzer, W. E., and Decker, T. S., *J. Bacteriol.,* **70,** 716–25 (1955)
111. Mitchell, P., and Moyle, J., *Biochem. J. (London),* **64,** 19P (1956)
112. Storck, R., and Wachsman, J. T., *J. Bacteriol.,* **73,** 784–90 (1957)
113. Alexander, M., *Bacteriol. Revs.,* **20,** 67–93 (1956)
114. Vennes, J. W., and Gerhardt, P., *Bacteriol. Proc.,* **105** (1957)
115. Klieneberger-Nobel, E., in McQuillen, K., *Symposium Soc. Gen. Microbiol.,* **6,** 127–49 (1956)
116. Klieneberger-Nobel, E., *Bacteriol. Revs.,* **15,** 77–103 (1951)
117. Dienes, L., and Weinberger, H. J., *Bacteriol. Revs.,* **15,** 245–88 (1951)
118. Tulasne, R., *Rev. immunol.,* **15,** 223–51 (1951)
119. Tulasne, R., in *Symposium: Bacterial Cytology,* Suppl. to *Rend. ist. super. sanitá,* 144–81 (1953)
120. Schellenberg, H., *Zentr. Bakteriol. Parasitenk. Abt. I(b) Orig.,* **161,** 433–57 (1954)
121. Freundt, E. A., *Acta Pathol. Microbiol. Scand.,* **27,** 159–71 (1950)
122. Bartmann, K., and Höpken, W., *Zentr. Bakteriol. Parasitenk. Abt I(b) Orig.,* **166,** 30–42 (1956)
123. Stempen, H., *J. Bacteriol.,* **70,** 177–81 (1955)
124. Taubeneck, U., *Zentr. Bakteriol. Parasitenk. Abt. I(b) Orig.,* **163,** 477–83 (1955)
125. Dienes, L., *Proc. Soc. Exptl. Biol. Med.,* **63,** 265–70 (1946)
126. Dienes, L., *J. Bacteriol.,* **57,** 529–46 (1949)
127. Klieneberger-Nobel, E., *J. Gen. Microbiol.,* **5,** 525–30 (1951)
128. Smith, W. E., Mudd, S., and Hillier, J., *J. Bacteriol.,* **56,** 603–18 (1948)
128a. Bringmann, G., *Z. Hyg. Infektions-krankh.,* **135,** 557–65 (1952)
129. Dienes, L., *J. Bacteriol.,* **66,** 280–86 (1953)
130. Kandler, G., Kandler, O., and Huber, O., *Arch. Mikrobiol.,* **21,** 202–16 (1954)
131. Klieneberger-Nobel, E., *Zentr. Bakteriol. Parasitenk. Abt. I(b) Orig.,* **165,** 329–43 (1956)
132. Rubio Huertos, M., and Desjardins, P. R., *Microbiol. españ.,* **9,** 375–82 (1956)
133. Pease, P., *J. Gen. Microbiol.,* **17,** 64–67 (1957)
134. Pease, P., *Giorn. microbiol.,* **3,** 44–45 (1957)
135. Dienes, L., Weinberger, H. J., and Madoff, S., *Proc. Soc. Exptl. Biol. Med.,* **75,** 409–12 (1950)
136. Poetschke, G., Killisch, L., and Uehleke, H., *Z. Immunitätsforsch.,* **114,** 406–15 (1957)
137. Sharp, J. T., Hijmans, M. D., and Dienes, L., *J. Exptl. Med.,* **105,** 153–59 (1957)
138. Kandler, O., and Zehender, C., *Z. Naturforsch.,* **12b,** 725–28 (1957)
139. Weibull, C., *Acta Pathol. Microbiol. Scand.,* **42,** 324–32 (1958)
140. Park, J. T., and Strominger, J. L., *Science,* **125,** 99–101 (1957)
140a. Weidel, W., and Primosigh, J., *Z. Naturforsch.,* **12b,** 421–27 (1957)
141. Park, J. T., and Johnson, M. J., *J. Biol. Chem.,* **179,** 585–92 (1949)

142. Park, J. T., *J. Biol. Chem.*, **194**, 877–904 (1952)
143. Strominger, J. L., Park, J. T., and Thompson, R., in Park, J. T., and Strominger, J. L., *Science*, **125**, 99–101 (1957)
144. Thompson, R., and Strominger, J. L., in Park, J. T., and Strominger, J. L., *Science*, **125**, 99–101 (1957)
145. Cooper, P. D., *Bacteriol. Revs.*, **20**, 28–48 (1956)
146. Prestidge, L. S., and Pardee, A. B., *J. Bacteriol.*, **74**, 48–59 (1957)
147. Nečas, O., *Nature*, **177**, 898–99 (1956)
148. Eddy, A. A., and Williamson, D. H., *Nature*, **179**, 1252–53 (1957)

# BACTERIAL VIRUSES[1]
## (With Particular Reference to Adsorption/Penetration)

By W. Weidel
*Max-Planck-Institut für Biologie,*
*Tübingen, Germany*

"It ain't Necessarily So!"[2]

Bacterial viruses have become subject to such varied studies that comprehensive reviews of the field as a whole have practically ceased to appear. Authors of more recent reviews (1 to 18) confine themselves to selected aspects, the multitude of which may seem an expression of the usual development of a chapter of science towards increasing specialization. But, in fact, the contrary is true here. Research on and with bacteriophages is not self-contained as may be the case with the systematics of leafhoppers. It is aimed at the solution of biologic and biochemical problems of the most general and the most far-reaching implications, namely: mode of codification of genetic information in nucleic acid molecules; mechanism of realization of genetic information carried by such polynucleotide chains; mechanism of their exact replication; and, finally, mechanism of mutation and genetic recombination at the molecular level.

The present article will touch upon the role of phage research in relation to these general problems, though its main purpose is, upon request, to pay particular attention to phenomena which at first sight appear to have much narrower bearing. Interest in analyzing the first phases of a phage particle's life cycle, adsorption to and penetration through the wall of the host cell, may betray the true specialist who is concerned with the peculiarities of his object rather than with its usefulness for generalizations.

Yet adsorption/penetration of a phage particle is just one example of how nature solves a ubiquitous problem: for biologic entities of all sorts, it is essential to establish specific and durable surface contacts to others as a prerequisite for uni- or bilateral exchanges of material or even for complete fusion. The infection mechanism of a phage particle is thus bearing the marks of what would be called a typically sexual process in more obvious cases.

The analogy is not only of a formal kind. Chemically defined macromolecular complexes serve as mediators for "sexual" attachment as well as for virus attachment. Whether these complexes are called receptor substances in the latter case or gamones in the first, functionally they must be considered as belonging to the same class.

[1] The survey of the literature pertaining to this review was concluded in October, 1957, with a few exceptions.

[2] See Preface.

Establishment of specific surface contacts is always a matter of two, possibly complementary, structures participating in the binding reaction. In the case of bacterial viruses, one is of bacterial origin, and the other is part of the anatomy of the phage particle. We begin with the first.

## The Receptor Mosaic of the *Escherichia coli* B-Cell Wall

If there is a bacterial structure capable of binding specifically phage particles of a given type, this structure must be a chemical component of the bacterial cell wall. If attempts to isolate such receptor-active wall material are to be successful, the binding reaction has to reach irreversibility *in vitro* as it does *in vivo*. Otherwise one would run into great technical difficulties in testing bacterial extracts for receptor activity. Fortunately, irreversibility of the *in vitro* reaction is encountered very frequently. For the mere purpose of extraction and purification of receptor substances, this fact can be gratefully accepted without searching deeper into the reasons why. They will be discussed later.

*Isolated cell walls.*—The *E. coli* B-system has been explored in this direction since 1949, when Weidel (19, 20) isolated B-cell walls in a form practically free from cytoplasmatic contents, and showed them to bind specifically and irreversibly phages of type T2, T4, T6, and, less readily, T3 and T7. Receptor activities against the rest of the T-series, which must have been present in the wall of the living cell, appeared to have been lost in the course of wall preparation.

This observation, and the pattern of partial phage resistance produced by mutations in the B-cell, suggested a mosaic-like structure of the wall [Weidel (21)]. Subsequently, dissection of the B-wall into different chemical components, arising along different biosynthetic pathways and carrying receptor activities against different phage types, proved to be feasible. Purified B-walls were shown by Weidel and co-workers to consist of at least two approximately concentric layers of very different chemical composition (22, 23, 24). The outside layer, amounting to about 80 per cent dry weight of the cell wall is made up of a plastic mixture of lipoprotein and some free lipide, whereas the building blocks of the rigid inside layer (20 per cent) are glucose, L-gala-D-manno-heptose (25), glucosamine, muramic acid (26), bound lipide, and a few characteristic amino acids. Both layers are separable by 90 per cent phenol which dissolves and removes the lipoprotein.

*Receptors for T3, T4, and T7.*—The different chemical composition of these layers is paralleled, as expected, by a different distribution of receptor activities among them. Activities against T3, T4, and T7 are all located in the 20 per cent layer, as can be observed directly by inactivation tests. This is in agreement with the well-known fact that there exists a one-step, B-mutant (B/3, 4, 7) unable to bind, and therefore resistant to, just these three phage types simultaneously, but not to others. The simplest explanation would be to assume that the mutant is incapable of synthesizing the 20 per cent layer in a way leading to the same structural configuration as in

wild-type cells. In fact, the structural change occurring in the multi-resistant mutant is rather drastic in this case: L-gala-D-manno-heptose was found to be missing entirely in the 20 per cent layer of B/3, 4, 7 (22). A very similar situation concerning the somatic antigen of *Shigella sonnei* was uncovered earlier by Goebel & Jesaitis (27, 28).

*Receptors for T2 and T6.*—Proof for the localization of T2- and T6-receptors is less direct so far. They are not detectable in the 20 per cent layer. Phenol treatment removes these receptors from the isolated B-wall, together with the wall-lipoprotein, though probably not in active form (22). Yet it seems most likely that they really are represented by two different components of the 80 per cent (lipoprotein) layer. Drastic chemical changes produced by mutation in the 20 per cent layer do not affect their activity, nor are the characteristic activities of the latter layer (T3, T4, T7) affected in turn by mutations to T2- or T6-resistance which are not even mutually inclusive.

*The T1-receptor.*—The mosaic of receptor-active structures composing the intact wall of the live B-cell would be completed, so far as the T-phages are concerned, by adding to the picture those responsible for binding T1 and T5. Unfortunately, nothing is known so far about the localization and chemistry of the T1-receptor, for the simple reason that this is the only one among the T-receptors whose binding reaction was never observed to reach irreversibility *in vitro*. Some unknown mechanism, apparently functioning in living cells only, must be indispensable for achieving irreversible binding of T1. As Weidel (21, 29) pointed out, this uniqueness of the T1-receptor system makes it unsuitable for drawing from its study any generalized conclusions about reversible attachment as the initial step of infection for all phage types [Puck, Garen & Cline (30)].

*The T5-receptor.*—The T5-receptor in turn is unique in other respects. The component of the B-wall carrying this activity is not integrated into the wall structure as rigidly as any of the others. With very weak alkali it is selectively extractable from fresh B-cells [Weidel (21, 31)]. Purification of the active material yielded a preparation of uniform particles of $11.4 \times 10^6$ mol. wt. [Weidel, Koch & Bobosch (32)]. In kinetic studies, each particle was shown to be capable of inactivating one, and only one, T5-particle. On electron micrographs this could be made directly visible. In T5-suspensions completely inactivated by adding an equivalent amount of T5-receptor, each T5-particle carries one of the spherical receptor particles firmly attached to the tip of its tail [Weidel & Kellenberger (33)].

In spite of their loose integration into the rest of the B-wall structure, the univalent T5-receptor particles mirror this structure in that they too are composed of two layers. Each particle of a diameter of *ca.* 30 mμ has a core of lipopolysaccharide, very similar in composition to that of the 20 per cent layer of the B-wall proper. Accordingly, the core, when exposed, shows receptor activity against T3, T4, and T7 known to be characteristic for such material. However, in the intact T5-receptor particle, the whole surface of

the core is completely covered by a layer of lipoprotein, rendering the core's own receptor areas inaccessible to T3, T4, or T7. Instead, the complete particle exhibits an exclusive binding capacity for T5, the site of which must therefore lie in the lipoprotein shell. Core and shell are separable by 90 per cent phenol, but, as in the case of T2- and T6-receptors, the T5-activity is not preserved after this treatment [Weidel *et al.* (32)].

From a mutant B/1,5, simultaneously resistant to T1 and T5, both of which do not attach to this cell type, it is possible to extract-material which in every respect resembles active T5-receptor very closely, except that the spherical particles of the mutant preparation are completely inert against T5 (32, 33). So far, chemical analysis has not revealed any differences, qualitative or quantitative, between active T5-receptor from B-cells and the inactive, biosynthetic analogue from B/1,5 [Koch & Weidel (34)]. Most amazingly, not even serologic methods permit discrimination between the two [Weidel & Koch (35)]. It must be concluded that the structural requirements which allow a phage particle to combine with its receptor are so exacting that the reaction fails even after changes at one of the participant surfaces too small to be detected with the most sensitive general method known for such purposes. This conclusion could not have been arrived at from the study of the B/3,4,7-case mentioned above, in which the change in the mutant structure is obvious enough to make one understand the concomitant loss of receptor activity.

Under these circumstances it becomes rather hard to believe that materials thoroughly different from specific bacterial receptors, like ion exchangers, are valuable "models" for them, although they seem to simulate some of their effects [Puck & Sagik (36)].

*Distribution and frequency of receptors in the wall.*—For two reasons it is difficult to estimate the numbers of receptors on the intact cell wall. First of all, the saturation experiments which are usually employed must yield underestimates in cases where the wall provides sites for more phage particles of a given type than can, for steric reasons, be actually accommodated at the same time. Secondly, irreversible attachment of at least some phage types causes severe structural changes in the cell wall itself, as will be discussed later. It cannot be excluded, therefore, that the wall, in the process of becoming saturated with phage, either exposes previously inaccessible sites or, instead, may be induced to do the opposite. This effect, and the first, could come into play especially with mixed-saturation experiments. Both may explain, for instance, why saturability with two different phage types used in sequence is often not reciprocal—that is, whether the second is still able to reach its own sites or not depends on which type goes on first [Weidel (21); Hershey & Chase (37); Watson (38)].

Under certain conditions it is possible to apply more clear-cut methods for determining the number of receptors per cell wall and the total area they cover. With the univalent receptor for T5 it could be shown that one kinetically defined "unit" of this material per ml. combines with T5-particles

at the same rate as $10^8$ B-cells per ml. Since the number of receptor particles per "unit" can be counted and was found to be approximately $10^{11}$, it follows that there are about $10^3$ T5-receptor particles imbedded into a single cell wall. The relation between the sum of their cross-sections and the total surface of the average coli cell indicates that these particles do not occupy more than 10 per cent of the latter [Weidel *et al.* (32)]. Clearly, the T5-receptor is too rare in the B-wall to permit a high efficiency of attachment for T5-particles after chance collisions with the cell. The comparatively low rate of adsorption for this phage type has long been known. Moreover, cells loaded with T5 can still rapidly adsorb T2, which indicates that the sites of attachment for T2 are much more abundant [Weidel (21)].

Combining all these results into a unitarian picture of the B-wall, one may arrive at the following conclusion. The rigid 20 per cent layer, carrying the receptor sites for T3, T4, and T7, is buried under a layer of plastic lipoprotein, but in a way which leaves part of these sites exposed [Weidel *et al.* (22)]. In other words, the lipoprotein layer, which represents an ocean of T2-sites with considerably less sites for T6 admixed in the form of a separate lipoprotein component, has holes or rather discontinuities in it through which many of the potential sites for T3, T4, and T7 remain accessible. In addition to the holes, it is dotted with comparatively few, loosely bound particles of their own individual make-up, carrying the sites for T5. Neither layer is rigidly bound to the other, e.g., by atomic bonds. It must be hydrogen bonds and the like which keep them together and may permit a gliding shift of their position relative to one another under certain conditions. In order to start its replication cycle, a phage particle must first deal with this enormously complex structure.

## Penetration

The problem is how to break through the imposing obstacle depicted above. For the T-even phages, the key to the problem appears to have been found. Weidel (20) showed on electron micrographs that T2-particles adsorbed to purified B-cell walls cause severe structural changes to occur in them. Considerable amounts of wall material are released, and the wall tends to disintegrate completely upon saturation with T2. The effect was ascribed to a lytic enzyme carried by the phage particles. Barrington & Kozloff (39, 40) confirmed the release of wall material after T2-adsorption by using walls labeled with isotopes.

The role of the suspected enzyme seemed obvious enough. It would render the phage particle capable of removing pieces of cell wall and, thereby, opening a thoroughfare for the injection of its "germinal substance," mostly DNA (deoxyribonucleic acid), into the interior of the cell—a process necessary for priming virus synthesis [Hershey (2); Hershey & Chase (37)].

The making of holes in the wall by phage particles, irreversibly bound to it, is indicated quite clearly in fact from observation of transient cellular

leakage which occurs immediately after mixing host cells and virus to start infection [Puck & Lee (41, 42)]. The suggestions of these authors on the mechanism of the leakage effect remained somewhat obscure though, because of their belief in autolytic cellular enzymes 'triggered" by phage attachment.

Under the original aspect of the directly acting phage enzyme, there is no difficulty in understanding another long-known phenomenon called "lysis from without" [Delbrück (43) and Doermann (44)]. The circumstances provoking it are either the simultaneous attack on the B-wall of a very large number of certain types of phage, especially T-even, or interference with the energy supply of the cell, when a much smaller number of phage particles suffices to disrupt the wall. Obviously then, lysis from without sets in whenever the damage done to the wall by a comparatively large, multiple dose of phage enzyme proceeds faster than the cell can repair it. The repair, by the way, if it can be done at all, apparently does not always reconstitute the original wall structure in every detail [Puck & Lee (42); Graham (45)]. One of the reasons may well be the delicate, positional equilibrium between the two wall layers which, when disturbed, can lead to extensive rearrangements. In their extreme, these are directly visible in the form of grotesque distortions and protrusion exhibited by cell walls heavily damaged by phage enzyme [Weidel (21)].

Finally, normal lysis at the end of the latent period would appear as just another manifestation of the phage enzyme, acting this time from within the infected cell where of course it must be synthesized in large quantity.

All of these earlier arguments in favour of a T-even enzyme are well supported by recent experimental developments. The primary question of which component or layer of the B-wall is attacked by the enzyme was answered by using the very handy technique of thoroughly labelling the wall with 2,4-dinitro-fluoro-benzene, which lends an intense yellow color to its components [Koch & Weidel (46)]. Split products from the wall, appearing after phage adsorption, can be readily measured photometrically and isolated for chemical analysis. Weidel & Primosigh (23, 24) found that the 20 per cent layer of the B-wall, obviously the main obstacle for penetration because of its mechanical strength, is indeed the "substrate" for the T-even enzyme which appears to have no type specificity, in contrast to the receptor-complementary sites of T2, T4, and T6 which determine their host range and are different from each other.

The point of attack of the enzyme, which is easily liberated from T2-particles by freezing and thawing (23), is indicated by chemical evidence to be a glycosidic bond. By breaking this special bond throughout the 20 per cent layer with a sufficient amount of free enzyme, the whole structure falls to pieces. The pieces are of two sorts: complexes of comparatively low molecular weight, and complexes still big enough to be sedimentable at 20,000 g. The complexes of the first kind are made up essentially from $\alpha,\varepsilon$-diaminopimelic acid, glutamic acid, alanine, muramic acid, and glu-

cosamine arranged in a peptidic chain. As a working hypothesis it is assumed that these chains serve as links for connecting the much larger complexes of the second kind to form a rigid network in which the latter are the knots [Weidel & Primosigh (24)]. These knots are composed of copolymerized glucose, L-gala-D-manno-heptose, phospholipides, and some glucosamine, and, most interesting, they carry the receptor sites for T3, T4, and T7 known to be characteristic for the 20 per cent layer. The action of the enzyme leaves these sites completely untouched.

A structure like the one outlined above for the 20 per cent layer is easy to perforate locally with an enzyme able to cut off the connecting links, provided the enzyme cannot move around on the wall too freely. In order to maintain a local effect, it must remain attached somehow to the tail of the phage particle which, in turn, is firmly bound to its receptor. Actually, there is direct evidence that the enzyme system carried by one phage particle is confined in its action to a limited area around the point of attachment. It can be shown that a single enzyme system is capable of splitting many more bonds in the wall than it does under normal conditions of infection [Koch & Weidel (46)].

In the case of multiple infection, it is quite clear that the holes can stay relatively small only as long as the areas attacked by the individual enzyme systems are still very far from overlapping. Whenever a few zones of enzyme action approach one another too closely, unproportionately large pieces of the wall structure must come loose all of a sudden. From here on, a complete breakdown of the structure is imminent, that is, lysis of the cell. To achieve it, there is obviously no need to first cut off every single connecting link. The observations of Puck & Lee (42) on the sequence—leakage of cytoplasmatic constituents of low molecular weight, followed by constituents of high molecular weight and eventually lysis, evoked by increasing multiplicity of infection—are thus explained.

A complete breakdown of the wall structure is desirable, if not indispensable, when the newly produced crop of phage particles has to be released from the cellular factory. This seems to be done by a surplus amount of free T-even enzyme produced in the infected cell, i.e., by enzyme not wrapped up in completed T-even particles. After normal lysis, much free enzyme is actually found in the lysate [Koch & Jordan (47)].

There is yet another observation to support this view. So-called "incomplete" lysogenic bacterial strains (48), if they can be induced at all to lyse, are then always found to have synthesized large quantities of free lytic enzyme which is presumably phage enzyme. It cannot become installed in complete phage particles because the prophage carried by the incomplete lysogenic strains lacks part or parts of the necessary "information" for making them. But the damaged prophage of inducible incomplete lysogenic strains still contains the information for the making of phage enzyme which it can still hand on to the cell for realization [Jacob (49)].

Within the T-series, not much is known about enzymatic activities of

phage types other than T2, T4, and T6 [cf. Barrington & Kozloff (40)]. Phages T3 and T7 may possess the same enzyme as T-even, though either in smaller quantity or more narrowly confined in action to the points of attachment of these phage particles. T1 and T5 may be closely related as to their modes of achieving penetration and liberation from the cell, but their enzymatic outfit, if there is any, is almost certainly different from that of all the others. Experimental evidence for making these suggestions comes from comparisons of lytic effects produced by the different T-types [Weidel 21)].

In concluding this section, it may be mentioned briefly that lysis of the B-cell as brought about by T-even enzyme, is very strikingly paralleled by lysis through the action of penicillin [Weidel & Primosigh (23, 24)]. This antibiotic, according to biochemical results of Park & Strominger (50), seems to prevent the cell from correctly incorporating into newly built pieces of cell wall just those connecting links of the 20 per cent layer which T-even enzyme can remove from the normal wall and which are indispensable for its mechanical strength. As a consequence, conspicuous morphologic changes of the cell are evoked. They are the same in both cases. However, the victims of these changes should not be called protoplasts [Lederberg (51); Mahler & Fraser (52); Zinder & Arndt (53)]. Their cytoplasm is still surrounded by the quantitatively predominant component of the original cell wall at least, namely, the lipoprotein layer. If this layer is no longer supported by a rigid frame, its plasticity causes it to yield to osmotic pressure, surface tension, electrostatic repulsion, etc., which force it into the form of a sphere and, if not counteracted, expand the sphere until it bursts.

## Functional Anatomy of Phage Particles

After the foregoing discussions, two questions are obvious, namely, which part of a phage particle is the receptor-complementary site serving for attachment, and where does the particle carry its enzyme for penetration (if it has any)?

In a general way, the first question has been answered by Anderson (54) who showed the "tail" to be the organ of attachment for phage particles. For T5, the very tip of the tail was demonstrated to be the site which combines irreversibly with the specific bacterial receptor. Combination with the receptor can cause the T5-particles to eject their DNA, but it is not at all clear how the ejection-mechanism is triggered, how it functions, and which way is taken by the DNA on escaping from the particle's head. The system offers no unambiguous hint at an intervening enzyme [Weidel *et al.* (32, 33)].

With the T-even phages, the situation is much more suggestive. Irreversible attachment of these phages to B-cells or to purified B-walls is always accompanied by striking changes occurring at the distal end of the phage tail [Kellenberger & Arber (55)]. Very thin filaments are unwound, but not disconnected, from the terminal half of the tail's whole length, thus

exposing a rod sticking out from the thicker proximal half of the tail. The filaments appear to mediate attachment by fastening to the cell wall. The rod may then puncture the wall and reach the cytoplasm, where it could be pulled out like a stopper from the proximal part of the phage tail to leave open a narrow channel through which the DNA might escape from the phage head straight into the interior of the cell [Kellenberger & Arber (55); Williams & Fraser (56); Kozloff & Lute (57)].

Taking into consideration other facts known about adsorption and penetration of the T-even phages this picture calls for specifications. Should one have to assume, for instance, that the tail filaments are, or carry, the receptor-complementary sites? Williams & Fraser (56) favour this view, but definite proof is lacking.

According to the reviewer's opinion, attachment to the receptor may occur, as in the case of T5, with the tip of the tail; and unwinding of the fibers may be the next step. Electron micrographs indicate that the fibers spread out on the cell wall to quite a distance from the tip of the phage tail to which they remain attached with one end (55). This would rather suggest them to be the carriers of the penetration enzyme, of which a considerable, yet limited, spread on the wall must be assumed, as explained earlier.

Kozloff offers still another interpretation. Experiments performed at his laboratory suggested to him that the proximal part of the tail should be the penetration enzyme [Brown & Kozloff(58)]. In order to explain how the enzyme under this condition can eventually make the indispensable contact with the wall, from which after primary attachment of the phage particle it still would be separated by half the tail's length, Kozloff revived the cellular enzyme hypothesis of Puck (59) in a new version. The cell wall is assumed to have a built-in enzyme whose main function in aiding penetration is to remove the impeding distal half of the tail. A wealth of most interesting information on agents capable of removing parts and pieces of T-even tails was brought forth to support the idea [Kozloff *et al.* (57, 60, 61)]. However, as was shown first by Jesaitis & Goebel (62), T4 can go through all the stages leading up to release of DNA from the phage head by reacting *in vitro* with its own specific receptor. Since this receptor-active material is thoroughly extracted with 90 per cent phenol in the course of its preparation, and thereby successfully freed from protein, it is not too probable that any enzyme could have survived this treatment.

It should be mentioned that one possibly separate function of the T-even tail is still completely in the dark as to the morphologic equivalent. Which agent is it that kills the cell when a particle of this type, or even its DNA-free protein ghost [Herriott (63)], attaches irreversibly to it? Is it the enzyme? It can kill, of course, when it actually lyses the cell [Koch & Jordan (47)]. But this is a trivial statement, and it seems clear that ghosts can kill without causing lysis. If the only function of the penetration enzyme were to remove a few of what are called the connecting links of the 20

per cent layer, it would be difficult to understand how this slight and reparable damage could cause the death of the cell. In any case, penicillin "protoplasts," with perhaps very little or no connecting links left, are not dead in any sense [Lederberg (51)]. If the enzyme is not the killing agent, then maybe it is the pin or rod of the T-even tail. In a biologic system still so near to chemistrylike phage, the role claimed for the pin as a purely mechanical device for puncturing the cell wall would appear a little too modest. A separate agent able to block some main metabolic pathway within the cell may be necessary to prepare it for phage synthesis in the normal course of infection [Hershey (4)].

As can be seen, the details of attachment and penetration mechanisms are still largely a matter of speculation. But in spite of the as yet tentative game of attributing functions, known to come into play here, to parts of the T-even tail known to be differentiable from others, the situation with T-even phages seems ripe for quick clarification just because of the many elements available now for integration.

When there is no clue for morphologic or other differentiation in the tail region, as in the case of T5, it is difficult to make similarly optimistic predictions. Yet this phage is useful for throwing light on one point which has remained a matter of controversy since it was made by Puck (59). Primarily on a theoretical basis, this worker visualized the specific binding which occurs between receptor and phage as being brought about by electrostatic forces distributed in complementary patterns on the two reacting sites. In other words, as the primary step of virus attachment some kind of salt formation should occur between negatively charged carboxyl groups on the wall and positively charged amino groups on the phage tailtip; and since salts are dissociable, this step had to be strictly reversible. In the sequence of events, the function of the receptor was supposed to end here. The specific binding through the receptor was never to become irreversible as such, but the ensuing release of DNA from the phage particle was to simulate irreversibility, so to speak, by making it impossible from then on to recover the intact phage particle.

At first sight the reaction between T5 and T5-receptor seems to fit Puck's idea very well. In a mixture of the two, about 50 per cent of the phage particles attached to receptor particles release their DNA into the medium, and they are of course "irreversibly attached" in Puck's sense. The other 50 per cent do not release their DNA and, according to Puck's hypothesis, they should not be inactivated—but they are! The bond formed between tailtip and receptor particle shows no tendency whatsoever to dissociate [Weidel *et al.* (32, 33)]. It must be concluded that the bond formation between receptor and phage is not necessarily reversible. It would be as interesting, as it is difficult, to find out what types of irreversible bonds are formed between the tailtip of T5 and the receptor particle. They must be of a peculiar kind since their formation means sensitizing the T5-particle for spontaneous ejection of its DNA.

Puck and his co-workers have done an enormous amount of work to prove their point [cf. Tolmach (13)], but there is actually not a single case to show unequivocally that reversible attachment precedes all further events in infection with phage. There are a few instances where, under quite unphysiologic conditions, reversible attachment of a phage to its host cell can be provoked. But it was never shown that reversibly bound phage, when conditions are restored to normal, can then proceed normally with infection as must be expected.

With T2, attempts in this direction have been made by Weidel (29). The result was distinctly negative. Upon return to conditions allowing normal, irreversible attachment when applied from the start, reversibly attached T2-particles were not only unable to infect the cell, they were actually lost! Hence, in the state of reversible attachment, their contact with the cell wall must have been quite different from the kind of contact leading to infection. In fact, Puck has obtained the same experimental result, as follows from a remark to be found in the review of Tolmach (13). The obvious conclusions should not have been ignored.

Hershey, who has taken up and extended these criticisms in a recent review (2), suggests a principally similar type of experiment with T1. It is not improbable that in this case one might have success with switching from reversible attachment to infection. As pointed out earlier, T1 is quite exceptional in that it is unable to attach irreversibly to anything but a living host cell. Perhaps of the T-series, T1 is the most "degenerate" phage and can do nothing more sophisticated than be attracted by straightforward electrostatic forces, leaving it entirely to the cell how to take any further steps. Nobody denies, of course, that Puck's type of receptor could exist, but even if so, it is almost certainly not the only one. A general theory of receptor function based on a few simple physicochemical assumptions is undoubtedly not warranted by present experimental evidence.

A really general aspect of the functional anatomy of phage particles was brought to light when results of the "blendor" experiment of Hershey & Chase (37) suggested the DNA of the particle ($2\times10^{-10}$ μg for T2) to be the infective agent par excellence, and reduced the protein part to the status of a protecting coat with attached devices for adsorption, penetration, injection, and other accessory functions [Hershey (2)]. The conclusion that the DNA alone might be the germinal substance of phage, containing the "genetic information" on all structural details of the particle, especially of the protein component or components, has focussed the attention of most phage workers on this puzzling compound.

The term genetic information which has come into frequent use, is somewhat misleading. The information to be handed on is most certainly not expressed in genuine "symbols" based on free choice in this case. On a higher biochemical level, there must exist as yet unknown but definite structural relations between DNA and protein, although to the organic chemist both seem entirely unrelated. The reliable handling of principally interchangeable

symbols to express and transmit information would require something like a central nervous system that can be taught and re-taught. This kind of flexibility cannot be expected of a cell which is so obviously bound to chemical laws.

## The Germinal Substance

Hershey (2) cautions against assuming without direct proof that nothing but, and all of, the DNA of a phage particle is the carrier of genetic specificity. In the case of T2, for instance, chemically the most thoroughly investigated phage, it is DNA of about 97 per cent purity which is injected into the host cell. This mixture of much DNA and little protein Hershey (64) calls the "germinal substance" of T2, to express due scepticism. Keeping this well in mind, DNA will still have to be treated as the sole carrier of genetic specificity since most workers in the field, including Hershey, find this hypothesis to be the most attractive, and argue accordingly.

The introduction to this review gave a brief outline of the research program on DNA under this aspect. Work on two of the four main issues of this program was greatly influenced and inspired by the suggestive DNA-model of Watson & Crick (65). It seemed to offer the long looked-for template mechanism for identical reproduction of material endowed with genetic specificity. It also correlated the wide variety of genetic information to be expressed (even in a phage!) with a practically infinite number of different nucleotide sequences to be constructed from only four different nucleotides forming long polynucleotide chains. It does not offer, however, any clear way to understand the mechanism of recombinant formation, and it gives no clue at all as to the steps involved in the "translation" of a specific nucleotide sequence into the finite phenotypic structure.

*Replication.*—Closer examination of the DNA model, together with new experimental evidence, revealed an ever-increasing number of difficulties which rendered even the assumed replication mechanism, not to speak of other functions, much less straightforward than it appeared at first. Delbrück & Stent (10) have recently summarized and discussed all known factors coming into play here. Their brilliant article should be consulted to appreciate the whole situation from the point of view of somebody giving great a priori credit to "the fundamental notion of replication by complementary pairing of purines and pyrimidines." A reader preoccupied in a different way and discouraged by a constant flow of ambiguous answers to apparently clearcut experiments designed for deciding the issue may ask himself eventually whether this pairing might not be just an ingenious way, for instance, of packing up those extremely long and vulnerable polynucleotide chains for storage. The complementary pairing would then betray as little about replication as does the sophisticated folding-up of a parachute betray how it functions when in actual use.

DNA in actual use may not be DNA at all any more [Stent *et al.* (66, 67); Tomizawa & Sunakawa (69)], but Hershey (2) calls it unjusti-

fied pessimism to assume that DNA-replication involves breakdown of parental DNA and resynthesis of offspring DNA from scrap [Kozloff (68)]. Under this aspect one would be left, of course, without the guidance of a model which keeps suggesting experiments and links so comprehensibly genetic continuity to material continuity. To be sure, there is as yet hope for eventual proof of the original concept. But still, quite often, it begins to look as if those favoring the untainted geometric mechanism of self-replication should at least be prepared for the worst.

Suspicions are raised especially by the protein which is built up early after infection and which is known to be indispensable for getting DNA-synthesis underway [Tomizawa & Sunakawa (69); Burton (70); Melechen (71)]. To upset the old scheme it would suffice to show that this protein steps into the shoes of parental DNA by taking over from there the information on how to turn out exact copies of just this kind of DNA. Early transfer of DNA-specificity on a structure practically insensitive to radiophosphorus decay—most probably protein—is clearly indicated by the exciting radiogenetic experiments of Stent (66) and may serve provisionally as indirect evidence for the specificity of the early protein. The question may be settled soon in a more direct way (3).

Orthodoxy, instead, must place all hope on being able to show that big pieces of parental DNA are really passed on undispersed over several generations of phage, and that these pieces keep containing and conferring always the same genetic specificity. This would be the clearest if not easiest way of proving her point [Hershey *et al.* (6); Stent *et al.* (10, 74); Levinthal *et al.* (72, 73)]. The final result of these efforts is still entirely at stake.

*Realization of genetic information.*—So far there is no direct experimental approach to unravelling the very first steps that are taken to translate genetic into phenotypic specificity. However, one circumstance seems clear now: synthesis of offspring DNA can proceed independently from synthesis of offspring protein coats—the specific end product of the translation process [Hershey *et al.* (3, 4, 75)]. If orthodoxy is right, this means that DNA molecules replicate in a "naked" state. They could not possibly do otherwise under this aspect. But since these offspring molecules do not seem to be responsible for the making of their individual coats (they may be put into the wrong ones!), who is? Undoubtedly there must occur some early branching of virus synthesis into two, from then on, independent pathways, each endowed once and for all with one of the two specificities the germinal substance has at its disposal (another early and decisive event!). Thinking of DNA as the only carrier of genetic specificity within the germinal substance, these observations could well mean that the infecting DNA is subjected to a treatment which is principally different from that which the offspring DNA experiences within the mother cell.

The orthodox theory of DNA-specificity and DNA-self-replication does not envision any such difference and plainly assumes the host cell can eat her cake and have it. Though directly supported by really not more than one

rather tricky experiment of Luria (76), it offers a constant temptation to explain away all the early events discovered so far. If they are taken to be more than mere side-effects, they could be combined by proposing the parental DNA to have but one task, and enough to do with it, namely: organizing two independently functioning sets of machinery, both represented by "early protein." One is for resynthesis of genetically specific DNA, the other for the manufacturing of equally specific coats. Biochemistry makes it most unlikely that a DNA-molecule could take care of this tremendous job and still survive intact.

There is other than indirect evidence suggesting that an independent machinery for the production of coats has to be developed early in the infected cell. Watanabe (77) found that phage coats can be made in very large amounts without concomitant synthesis of phage DNA. The full establishment of this machinery seems to take more time than is needed for the DNA-producing set to function and may determine the length of the eclipse period as well as the period which is needed for the infected cell to reach full radio stabilization.

*Maturation.*—The two pathways of virus synthesis come together again when a complete phage particle is to be formed from coat and DNA. But, very characteristically, this final step is not rigidly directed by genetic specificity! If it were, phenotypic mixing could not occur [Streisinger (78); Brenner (79)]. Unfortunately, present knowledge about the site of phenotypic mixing is restricted to the tail structure. It would be of greatest interest to see whether the head protein, which is in direct contact with the particle's DNA, is also freely interchangeable. One wants to know whether everything concerning specificity is decided upon early and, therefore, by the injected germinal substance alone; and whether the copies of it, which are waiting for eventual incorporation into new phage particles, are about as inert within the mother cell as these are known to be (11).

It remains to be clarified how coat and DNA are put together. All one knows is how to prevent the completing step called maturation. It can be done with proflavin [De Mars (80); Kellenberger & Séchaud (81)].

*Participation of RNA in virus synthesis.*—Since evidence is accumulating from everywhere that protein synthesis requires the participation of RNA (ribonucleic acid) and since in the infected cell there is "early protein" to be made as well as protein coats, the existence of some virus specific RNA-metabolism is very likely. It would, of course, further complicate a clear understanding of the functioning of the Watson-Crick model. To account on its basis for a possible intervention of RNA between parental and offspring DNA, Stent (12) has devised a modification of the original model. It has the advantage of explaining at the same time how genetic recombination could occur at the molecular level and how the genetic information could be transferred from the parental DNA-duplex upon something more stable to radiation effects. The trick is done, according to Stent, by the in-

tervention of ribonucleoprotein chains which form in the groove offered by the DNA double-helix, copy its specific nucleotide sequence by using another complementary pairing code, pair or "mate" among themselves to form their own kind of duplexes, and thus permit the synthesis of progeny DNA duplexes in a reversion of the process which led to their own formation. All this requires endless "speedometer-cable" turning [Levinthal & Crane (82)] to untwist the necessarily intertwined primary or intermediate products of synthesis, but with plectonemic coils for templates one must not shirk such measures.

For the reviewer, the question has always remained a cause of wonder whether the obvious advantages of sequences over templates for storing and transmitting a multitude of information are really ignored in nature to such an extent that a sequence, even if at hand, is degraded to function as a mere template (83). The exclusive advantage of the sequence lies in the fact that, in a dynamic system, it permits making use of time as an additional parameter. The living cell is a dynamic system and should be able to read the news off a polynucleotide helix by removing from it the nucleotides bit by bit as they follow one another from end to end, without necessarily "forgetting" about the sequence of those which were already removed. Some of the assumptions needed to make the DNA double-helix work as a template have become by themselves improbable enough, it would seem, to justify an effort to escape them altogether, if only by substituting new improbabilities for old ones. The field is not at all closed to a little exercise in free imagination.

To return to firmer ground, this paragraph may be closed by mentioning some biochemical observations which, though not yet thoroughly explored, might really indicate an important role for RNA to play in virus synthesis. Volkin & Astrachan (84, 85) detected some net synthesis of RNA in T2-infected bacteria. This RNA differs from normal bacterial RNA in its purine-pyrimidine ratio and remains different throughout the reproductive cycle of the phage. Hershey (2) has evidence of a metabolically active pool formed by this RNA in the T2-infected cell, amounting to less than 10 per cent of the total intrabacterial RNA. On stopping protein synthesis with chloramphenicol, RNA piles up in this pool. With protein synthesis setting in again after removing the antibiotic, the surplus RNA in turn is removed from the pool and broken down—a result which points to a correlation of protein synthesis and RNA-breakdown. It cannot be overlooked that with this assumption utter heresy is lurking in the background. After all, specific nucleotide sequences might then be translated into specific protein structures by using them as sequences in the sense discussed above, and not as templates. For viruses containing RNA instead of DNA to carry and confer genetic specificity, one does not know anyway how to apply the principle of complementary purine-pyrimidine pairing for explaining replication [Rich & Watson (86)].

## Genetics at the Molecular Level

There is no direct way as yet of determining the actual nucleotide sequence in specific RNA or DNA strands [Markham (87)]. For obvious reasons, purification and stepwise chemical degradation are much more problematic for polynucleotide chains than for polypeptides or even proteins. Undoubtable proof of the really fundamental notion of specific nucleotide sequences as the chemical equivalent of genetic specificity is thus still lacking. Once the technical difficulties are overcome, the way will be opened for attempts to relate phenotypic to genetic structures [Streisinger (88)], though even then the key to the code may remain obscure for a long time. It is unknown whether there is any virus at all which really carries in its genetic structure the complete equivalents for all of its component structures. Virus reproduction may always call for the host cell to step in with supplements of some kind or other definitely exceeding a mere lease of, say, its energy supply system [cf. Garen & Zinder (89)]. For the same reason, nobody would expect the structure of capsular polysaccharide in pneumococci to have its complete, decipherable equivalent in the structure of the transforming DNA.

At the present time, there is only one technique available for probing into details of the linear arrangement of genetic determinants, but this technique seems unpleasantly dependent on chance circumstances determining its power of resolution, and it yields but relative values for lengths and distances. Its basis is the phenomenon of genetic recombination, and it is one of the great discoveries in phage work that this phenomenon is not restricted to chromosomal genetics.

The resemblance of effects observed in phage genetics and in chromosomal genetics has turned out more and more to be rather superficial, and we are forced by now to consider as inadequate all attempts to take mechanisms of chromosomal genetics too directly as a basis for interpreting the process leading to genetic recombination in phage. Such attempts culminated in the mating theory of Visconti & Delbrück (90), which treated vegetative phage as a pool of naked, self-replicating DNA-strands subject to random pairings and crossovers in the classical sense. By thus superimposing population genetics over chromosome genetics, a quantitative description of some major effects in phage genetics was achieved for the first time. More recent experimental evidence, tending to exclude anything like classical crossover as the mechanism producing recombinants in phage, comprises the following: the formation of heterozygotes [Hershey & Chase (91); Jacob & Wollman (92); Levinthal (93); Bresch (94)]; the nonreciprocity of recombination (92, 94) [Kaiser (95)]; the frequent formation of double recombinants in what appears as one mating act (95) [Jacob & Wollman (96); Stent (97); Stahl (98)]; and the introduction of asymmetry into crosses by participation of one irradiated parental type (96).

A template model or rather a scheme avoiding the difficulties created for the classical one by these findings can still be constructed. It is called the

partial replica or copying choice mechanism [Hershey (99); Lederberg (100); Levinthal (73, 93); Bresch (94)]. Since there is good reason to believe that recombination and growth of vegetative phage are but two aspects of the same process (12, 93, 94, 96), a clear decision in favor of the copying choice model may be expected from biochemical studies on phage growth rather than from phage genetics itself. Unfortunately, there is just one of many principally possible experimental results to be foreseen as unambigously interpretable, namely, clear evidence for the material integrity of the genetically specific parental template throughout a succession of many rounds of replication and recombination (10).

With so many excellent reviews available on these implications of genetic recombination in phage (6, 11, 12, 101, 102) it is not necessary to go into the details once more. Instead, we will return to the use of phage genetics as a tool which can bring to light "the elementary units of heredity" [Benzer (103, 104, 105)]. This ambitious program requires that the tool turn into a microscope which would permit in principle the measurement of map distances between genetic markers definitely smaller than could occur, for theoretical reasons, in reality.

What is the theoretical limit? Assuming one long double helix of DNA, composed of nucleotide pairs in specific sequences, to be the phage "chromosome," recombination between two such strands could obviously not subdivide this linear structure into less than one nucleotide pair. The DNA of a T-even phage contains $2 \times 10^5$ nucleotide pairs which would have to be related to the total length of the linkage map of the particle, in order to get a rough estimate of the per cent recombination per nucleotide pair. For T4, the map length could be taken to be 200 recombination units. By division one finds $10^{-3}$ recombination units ($= 10^{-3}$ per cent recombination) per pair. In other words, a cross between two phages, carrying markers just one nucleotide pair apart, would yield one recombinant particle in $10^5$. This is the order of magnitude for the lower limit of map distance to be expected on the premises of the assumptions made.

The equivalent of the "microscope" would, therefore, have to be a screening method allowing detection of much less than one recombinant in $10^5$. Benzer has a method enabling him to detect one in $10^8$! It turned out that the lowest frequency of recombinants actually observed is only $10^{-4}$. This could mean either that recombination does not subdivide DNA into units of length ("recons") shorter than about a dozen nucleotide pairs in a row, or, if separation were to occur between single pairs of nucleotides, that not all of the phage DNA is genetically specific. A similar estimate of the shortest length that has to be altered in order to produce a mutant ("muton"), yielded a maximum of five nucleotide pairs. The units of length comprising and exerting some kind of integrated functions ("cistrons") must be of a very much larger size—about 4000 nucleotide pairs. These functions can be impaired by mutations at many different locations along the entire length of such pieces.

It is interesting to note that two DNA strands carrying a mutation each at two different locations within the same cistron cannot always repair the damage by recombination, supposedly because they cannot even start to multiply. This is not to be foreseen and difficult to explain on the basis of the orthodox theory of replication and mating of naked DNA strands. One may as well take it as still another indication of the specificity and importance of "early events" in phage multiplication. If large pieces of un- on having reached at last that ground level, long hidden under clouds of of parental DNA into something else before the structure can be copied, it must be very far from the autonomy envisioned for self-replicating and freely mating DNA strands.

With Benzer's observations and concepts, phage research can pride itself on having reached at last that ground level, long hidden under clouds of speculation, where the geneticist's language merges with ease into the language of the chemist and thereby rids itself of the relativity of its quantitative assertions.

## Lysogeny and Transduction

From here on the way turns back into realms where biologic rather than chemical terminology is still best suited for analyzing the facts. The basic notions of this peculiar field of work with phage, in which phages appear in the disguise of normal cell constituents and normal cell constituents disguised as phage, have been reviewed repeatedly during the last years by those most competent to do so (14, 17, 106). Therefore, making a few remarks and raising a few questions may suffice.

For example, it should be noted that the very existence of phage as prophage provides in itself a good enough illustration for the dissociability of the copying mechanism for specific DNA from the mechanism translating the information on specific protein synthesis carried by it. One can function without the other. For the virulent phage T2 it took very laborious experiments with chloramphenicol to show the same (75).

What causes prophage DNA in the lysogenic cell to renounce half of its rights? Some very early step in the sequence of reactions leading to synthesis of specific phage protein must be blocked. This step is unknown, but, for ultraviolet-inducible prophages at least, some remarkable facts have come to light which seem to indicate that the block can merely be kept up, not directly created by the prophage. If an ultraviolet-inducible prophage enters, as such, a nonlysogenic cell, not in the form of a temperate phage via normal infection, the production of mature phage particles sets in immediately, and the cell lyses. Consequently, there seems only one way of initiating the block: it has to be done in the course of those fleeting preparatory steps along which the temperate phage is reduced after infection to the state of prophage. History and nuclear constitution together create the final equilibrium of lysogeny in the cell.

This idea is well supported by the observations of Levine (107, 108) and

Kaiser (109) on mutual complementariness of individually virulent phage types which can help each other to establish the block in mixed infection but are incapable of having themselves reduced into prophages when infecting alone. In the latter case the cell turns directly into the lytic cycle and produces new phage particles, whereas in the former it becomes lysogenic for one of the two virulent phages, or even for both. Once in the state of prophage, they can now easily and independently keep up the block they were unable to bring about except by temporary collaboration. The key to explaining the situation may be that the reaction to be blocked is governed by pieces of just that segment of the bacterial chromosome where ultraviolet-inducible prophages have to attach themselves eventually. Preparations for attachment of the corresponding site may initiate the block which is later on kept up simply through something like steric hindrance caused by the attached prophage.

There are two ways of transferring prophage as prophage from cell to cell. A genetic cross between bacteria is one of them. If the recipient cell lyses immediately, as in the case mentioned above, one speaks of "zygotic induction" [Jacob & Wollman (17)]. The second way is to make use of the strangest of all permutatory combinations conceivable on this artistic playground of nature and biologists: a prophage in the disguise of a phage. In other words, lysogeny can be transduced [Lennox (110); Jacob (111)]. The recipient cell can produce phage at once and lyse—a parallel to zygotic induction—but lysis may be prevented and the transduced prophage established in its new surroundings by lowering the temperature. Definite establishment of the prophage requires substitution by recombination of the transduced chromosomal segment with attached prophage for the homologous prophage-free piece owned by the recipient cell. If substitution is fast, it may outrun the onset of the lytic response, the first step of which was thought, as intimated above, to be governed by the prophage-free, unblocked piece of segment. Temperature could well decide which goes faster.

But a reviewer ought not to be tempted by his subject matter, even if it owes a very great deal of its attraction to aptness for constructive speculation, to indulge in probably quite unwarrantable hypothesis-making of his own. He retreats with apologies.

## LITERATURE CITED

1. Hershey, A. D., *Currents in Biochemical Research,* 1–28 (Green, D. E., Ed., Interscience Publishers, Inc., New York, N.Y., 1956)
2. Hershey, A. D., *Advances in Virus Research,* **4,** 25–61 (1957)
3. Hershey, A. D., *Enzymes: Units of Biological Structure and Function,* 109–17 (Academic Press Inc., New York, N.Y., 1957)
4. Hershey, A. D., *Mutation. Brookhaven Symposia in Biol.,* **8,** 6–14 (1956)
5. Hershey, A. D., *Harvey Lectures,* **LI,** 229–39 (1957)
6. Hershey, A. D., and Burgi, E., *Cold Spring Harbor Symposia Quant. Biol.,* **21,** 91–101 (1956)

7. Luria, S. E., *Cellular Metabolism and Infections,* 132–37 (Racker, E., Ed., Academic Press, Inc., New York, N.Y., 1954)
8. Lwoff, A., *Harvey Lectures,* **L,** 92–111 (1954–55)
9. Herriott, R. M., *The Chemical Basis of Heredity,* 399–407 (McElroy, W. D., and Glass, B., Eds., The Johns Hopkins Press, Baltimore, Md., 848 pp., 1957)
10. Delbrück, M., and Stent, G. S., *The Chemical Basis of Heredity,* 699–736 McElroy, W. D., and Glass, B., Eds., The Johns Hopkins Press, Baltimore, Md., 848 pp., 1957)
11. Doermann, A. H., Chase, M., and Stahl, F. W., *J. Cellular Comp. Physiol.,* **45,** Suppl. 2, 51–74 (1955)
12. Stent, G. S., *Advances in Virus Research,* **5,** 95–149 (1958)
13. Tolmach, L. J., *Advances in Virus Research,* **4,** 63–110 (1957)
14. Lwoff, A., *Bacteriol. Rev.,* **17,** 270–337 (1953)
15. Jacob, F., *Les bactéries lysogènes et la notion de provirus* (Masson, Paris, 176 pp., 1954)
16. Bertani, G., *Advances in Virus Research,* **5,** 51–93 (1958)
17. Jacob, F., and Wollman, E. L., *The Chemical Basis of Heredity,* 468–98 (McElroy, W. D., and Glass, B., Eds., The Johns Hopkins Press, Baltimore, Md., 848 pp., 1957)
18. Bertani, G., *Mutation. Brookhaven Symposia in Biol.,* **8,** 50–56 (1956)
19. Benzer *et al., Viruses, 1950,* 119–21 (Delbrück, M., Ed., Div. of Biology, Calif. Institute of Technology, Pasadena, Calif., 147 pp., 1950)
20. Weidel, W., *Z. Naturforsch.,* **6b,** 251–59 (1951)
21. Weidel, W., *Ann. inst. Pasteur,* **84,** 60–65 (1953)
22. Weidel, W., Koch, G., and Lohss, F., *Z. Naturforsch.,* **9b,** 398–406 (1954)
23. Weidel, W., and Primosigh, J., *Z. Naturforsch.,* **12b,** 421–27 (1957)
24. Weidel, W., and Primosigh, J., *J. Gen. Microbiol.,* **18,** 513–17 (1958)
25. Weidel, W., *Z. physiol. Chem., Hoppe-Seyler's,* **299,** 253–57 (1955)
26. Strange, R. E., *Biochem. J.,* **64,** 23P (1956)
27. Jesaitis, M. A., and Goebel, W. F., *J. Exptl. Med.,* **96,** 409–24 (1952)
28. Goebel, W. F., and Jesaitis, M. A., *J. Exptl. Med.,* **96,** 425–38 (1952)
29. Weidel, W., *Cold Spring Harbor Symposia Quant. Biol.,* **18,** 155–57 (1953)
30. Puck, T. T., Garen, A., and Cline, J., *J. Exptl. Med.,* **93,** 65–88 (1951)
31. Weidel, W., *Atti congr. intern, microbiol, 6th Congr.,* **3,** X, 458–61 (1953)
32. Weidel, W., Koch, G., and Bobosch, K., *Z. Naturforsch.,* **9b,** 573–79 (1954)
33. Weidel, W., and Kellenberger, E., *Biochim. et Biophys. Acta,* **17,** 1–9 (1955)
34. Koch, G., and Weidel, W., *Z. physiol. Chem., Hoppe-Seyler's,* **303,** 213–23 (1955)
35. Weidel, W., and Koch, G., *Z. Naturforsch.,* **10b,** 694–98 (1955)
36. Puck, T. T., and Sagik, B., *J. Exptl. Med.,* **97,** 807–26 (1953)
37. Hershey, A. D., and Chase, M., *J. Gen. Physiol.,* **36,** 39–56 (1952)
38. Watson, J. D., *J. Bacteriol.,* **60,** 697–718 (1950)
39. Barrington, L. F., and Kozloff, L. M., *Science,* **120,** 110–11 (1954)
40. Barrington, L. F., and Kozloff, L. M., *J. Biol. Chem.,* **223,** 615–27 (1956)
41. Puck, T. T., and Lee, H. H., *J. Exptl. Med.,* **99,** 481–94 (1954)
42. Puck, T. T., and Lee, H. H., *J. Exptl. Med.,* **101,** 151–75 (1955)
43. Delbrück, M., *J. Gen. Physiol.,* **23,** 643–60 (1940)
44. Doermann, A. H., *J. Bacteriol.,* **55,** 257–76 (1948)

45. Graham, A. F., *Ann. inst. Pasteur,* **84,** 90–98 (1953)
46. Koch, G., and Weidel, W., *Z. Naturforsch.,* **11b,** 345–53 (1956)
47. Koch, G., and Jordan, E. M., *Biochim. et Biophys. Acta,* **25,** 437 (1957)
48. Jacob, F., *Ann. inst. Pasteur,* **90,** 282–302 (1956)
49. Jacob, F., and Fuerst, C. R., *J. Gen. Microbiol.,* **18,** 518–26 (1958)
50. Park, J. T., and Strominger, J. L., *Science,* **125,** 99–101 (1957)
51. Lederberg, J., *Proc. Nat. Acad. Sci. U.S.,* **42,** 574–77 (1956)
52. Mahler, H. R., and Fraser, D., *Biochim. et Biophys. Acta,* **22,** 197–99 (1956)
53. Zinder, N. D., and Arndt, W. F., *Proc. Natl. Acad. Sci. U.S.,* **42,** 586–90 (1956)
54. Anderson, T. F., *Cold Spring Harbor Symposia Quant. Biol.,* **18,** 197–203 (1953)
55. Kellenberger, E., and Arber, W., *Z. Naturforsch.,* **10b,** 698–704 (1955)
56. Williams, R. C., and Fraser, D., *Virology,* **2,** 289–307 (1956)
57. Kozloff, L. M., and Lute, M., *J. Biol. Chem.,* **228,** 537–46 (1957)
58. Brown, D. D., and Kozloff, L. M., *J. Biol. Chem.,* **225,** 1–10 (1957)
59. Puck, T. T., *Cold Spring Harbor Symposia Quant. Biol.,* **18,** 149–54 (1953)
60. Kozloff, L. M., Lute, M., and Henderson, K., *J. Biol. Chem.,* **228,** 511–28 (1957)
61. Kozloff, L. M., and Lute, M., *J. Biol. Chem.,* **228,** 529–36 (1957)
62. Jesaitis, M. A., and Goebel, W. F., *Cold Spring Harbor Symposia Quant. Biol.,* **18,** 205–8 (1953)
63. Herriott, R. M., *J. Bacteriol.,* **61,** 752–54 (1951)
64. Hershey, A. D., *Virology,* **1,** 108–27 (1955)
65. Watson, J. D., and Crick, F. H. C., *Cold Spring Harbor Symposia Quant. Biol.,* **18,** 123–31 (1953)
66. Stent, G. S., *J. Gen. Physiol.,* **38,** 853–65 (1955)
67. Stent, G. S., and Fuerst, C. R., *Virology,* **2,** 737–52 (1956)
68. Kozloff, L. M., *Cold Spring Harbor Symposia Quant. Biol.,* **18,** 209–19 (1953)
69. Tomizawa, J., and Sunakawa, S., *J. Gen. Physiol.,* **39,** 553–65 (1956)
70. Burton, K., *Biochem. J.,* **61,** 473–83 (1955)
71. Melechen, N., *Genetics,* **40,** 584 (1955)
72. Levinthal, C., *Proc. Natl. Acad. Sci. U.S.,* **42,** 394–404 (1956)
73. Levinthal, C., and Thomas, Jr., C. A., *The Chemical Basis of Heredity,* 737–43 (McElroy, W. D., and Glass, B., Eds., The Johns Hopkins Press, Baltimore, Md., 848 pp., 1957)
74. Stent, G. S., and Jerne, N. K., *Proc. Natl. Acad. Sci. U.S.,* **41,** 704–9 (1955)
75. Hershey, A. D., and Melechen, N. E., *Virology,* **3,** 207–36 (1957)
76. Luria, S. E., *Cold Spring Harbor Symposia Quant. Biol.,* **16,** 463–70 (1951)
77. Watanabe, I., *J. Gen. Physiol.,* **40,** 521–31 (1957)
78. Streisinger, G., *Virology,* **2,** 388–98 (1956)
79. Brenner, S., *Virology,* **3,** 560–74 (1957)
80. De Mars, R. I., *Virology,* **1,** 83–99 (1955)
81. Kellenberger, E., and Séchaud, J., *Virology,* **3,** 256–74 (1957)
82. Levinthal, C., and Crane, H. R., *Proc. Natl. Acad. Sci. U.S.,* **42,** 436–38 (1956)
83. Weidel, W., *Lehrbuch der physiologischen Chemie,* **II** (Flaschenträger, B., and Lehnartz, E., Eds., Springer-Verlag, Heidelberg, Germany, in press)
84. Volkin, E., and Astrachan, L., *Virology,* **2,** 149–61 (1956)

85. Volkin, E., and Astrachan, L., *The Chemical Basis of Heredity*, 686–94 (McElroy, W. D., and Glass, B., Eds., The Johns Hopkins Press, Baltimore, Md., 848 pp., 1957)
86. Rich, A., and Watson, J. D., *Proc. Natl. Acad. Sci. U.S.*, **40**, 759–64 (1954)
87. Markham, R., *Cold Spring Harbor Symposia Quant. Biol.*, **18**, 141–48 (1953)
88. Streisinger, G., *Virology*, **2**, 377–87 (1956)
89. Garen, A., and Zinder, N. D., *Virology*, **1**, 347–76 (1955)
90. Visconti, N., and Delbrück, M., *Genetics*, **38**, 5–33 (1953)
91. Hershey, A. D., and Chase, M., *Cold Spring Harbor Symposia Quant. Biol.*, **16**, 471–79 (1951)
92. Jacob, F., and Wollman, E. L., *Ann. inst. Pasteur*, **87**, 653–91 (1954)
93. Levinthal, C., *Genetics*, **39**, 169–84 (1954)
94. Bresch, C., *Z. Naturforsch.*, **10b**, 545–61 (1955)
95. Kaiser, A. D., *Virology*, **1**, 424–43 (1955)
96. Jacob, F., and Wollman, E. L., *Ann. inst. Pasteur*, **88**, 724–49 (1955)
97. Stent, G. S., *Proc. Natl. Acad. Sci. U.S.*, **39**, 1234–41 (1953)
98. Stahl, F. W., *Virology*, **2**, 206–34 (1956)
99. Hershey, A. D., *Intern. Rev. Cytol.*, **1**, 119 (1952)
100. Lederberg, J., *J. Cellular Comp. Physiol.*, **45**, Suppl. 2, 75–107 (1955)
101. Doermann, A. H., *Cold Spring Harbor Symposia Quant. Biol.*, **18**, 3–11 (1953)
102. Hershey, A. D., *Advances in Genet.*, **5**, 89–106 (1953)
103. Benzer, S., *The Chemical Basis of Heredity*, 70–93 (McElroy, W. D., and Glass, B., Eds., The Johns Hopkins Press, Baltimore, Md., 848 pp., 1957)
104. Benzer, S., *Proc. Natl. Acad. Sci. U.S.*, **41**, 344–54 (1955)
105. Benzer, S., *Mutation. Brookhaven Symposia in Biol.*, **8**, 3–5 (1956)
106. Bertani, G., *Cold Spring Harbor Symposia Quant. Biol.*, **18**, 65–70 (1953)
107. Levine, M., *Genetics*, **40**, 582 (1955)
108. Levine, M., *Virology*, **3**, 22–41 (1957)
109. Kaiser, A. D., *Virology*, **3**, 42–61 (1957)
110. Lennox, E. S., *Virology*, **1**, 190–206 (1955)
111. Jacob, F., *Virology*, **1**, 207–20 (1955)

# NEWLY RECOGNIZED RESPIRATORY TRACT VIRUSES[1,2]

By Robert J. Huebner, Wallace P. Rowe, and Robert M. Chanock
*Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare, Bethesda, Maryland*

## Introduction

Perhaps one of the most significant developments arising out of recent advances in research of viral disease is the realization that the common, undifferentiated respiratory diseases represent extremely varied and complex problems. The somewhat frenetic preoccupation with searches for "the common cold virus" has been generally replaced by more sophisticated efforts which, perhaps, have been best described in a recent symposium entitled *Viruses in Search of Disease* (1). It is now quite apparent that rather similar respiratory illnesses have multiple and, perhaps, numerous viral causes, and that the same or similar respiratory viruses can produce variable clinical manifestations. Thus, the newly recognized adenoviruses (2), CA (croup-associated) (3), respiratory syncytial [chimp coryza agent (CCA)] (4, 5), JH (6), 2060 (7), and the new HA (hemadsorption) (8) agents are frequently demonstrated in upper respiratory secretions, and some have been shown to account for substantial but, as yet, undetermined amounts of respiratory illness. Much remains to be elucidated about the roles of these viruses as etiologic agents, and it is clear that a large amount, possibly a majority, of respiratory diseases still remain unexplained.

The purpose of this review is to assemble available information on the newly recognized viral agents which are classified as respiratory viruses, chiefly because they are demonstrable in the respiratory systems of persons with acute respiratory disease. Respiratory illnesses of well-known etiology such as influenza, including the recent Asian Type A, will not be reviewed. Certain representatives of the enteroviruses (9) cause clinical signs and symptoms in the respiratory tract (herpangina, summer pharyngitis, "summer fevers,' and "nonparalytic poliomyelitis"), (10) chiefly in the warm months, but they are regarded as separate subjects deserving extensive reviews in their own right.

[1] The survey of the literature pertaining to this review was concluded in February, 1958.

[2] The following abbreviations are used: APC (adenoidal-pharyngeal-conjunctival); ARD (acute respiratory disease); CA (croup-associated); CCA (chimpanzee coryza agent); CF (complement-fixing); DNA (deoxyribonucleic acid); EKC (epidemic keratoconjunctivitis); HA (hemadsorption); H-I (hemagglutin-inhibitory); PAP (primary atypical pneumonia); RDE (receptor destroying enzyme); RI (respiratory illness).

## The Adenoviruses

The adenoviruses comprise a large family of agents which have been demonstrated in the respiratory, ocular, and enteric systems of man and some of the higher primates. At least 18 separate serotypes are known to affect man, and no less than 6 distinguishable clinical syndromes are attributable to certain of these agents (11). These clinical syndromes affect chiefly the respiratory and ocular systems, and include acute respiratory disease (ARD) of military recruits, nonbacterial pharyngitis (febrile pharyngitis) in children, pharyngoconjunctival fever, follicular conjunctivitis, keratoconjunctivitis, and viral pneumonitis (atypical pneumonia without cold agglutinins).

In addition to fever, the adenoviruses characteristically produce catarrhal inflammation of the mucous membranes of the respiratory and ocular systems, accompanied by follicular hyperplasia of submucous lymphoid tissues and enlargement of the regional lymph nodes (12). In more severe but less common adenoviral illnesses such as infant pneumonitis [inclusion-body type described by Goodpasture *et al.* (13)], occasional fatalities may occur (14,15). Severe epidemic keratoconjunctivitis may be followed by permanent damage to the cornea. On the whole, however, adenoviral illnesses are usually self-limited and seldom fatal (11).

Epidemiologically, the viruses can be divided into the extremely prevalent endemic types which occur chiefly in early childhood (types 1, 2, and 5), (11), the epidemic types that have been observed in military recruits (chiefly types 4 and 7), (16), and types such as 3, 7a, and 14 which have been observed causing community outbreaks (17 to 20). Certain undetermined factors influence the prevalence of different types of adenoviruses, serologic surveys revealing different patterns of antibodies in different parts of the world (21).

Historically considered, the current information about the adenoviruses and the diseases they cause represents a prime example of the application of modern tissue-culture techniques to rapid delineation of viral disease problems, and their prevention. The initial reports in 1953 and 1954 which described the adenoviruses, first as masked infections of the adenoids and tonsils [Rowe *et al.* (22)], then as etiologic agents of ARD and atypical pneumonia in military recruits [Hilleman & Werner (23)], triggered extensive studies of the attributes of the new agents as well as their roles as causes of disease in civilian and military populations. Later in 1954, Huebner *et al.* (24) reported 143 strains of viruses having similar biologic properties, segregated them into six separate immunologic types, and proposed a schema for their classification. The family of viruses was named adenoidal-pharyngeal-conjunctival (APC), after the principal anatomic sites in which the agents were found. Subsequently, in order to resolve the confusion deriving from the use of several different terms [RI (23), ARD (18), and APC (24)], the term "adenovirus" was agreed upon and is now in general use (2). Two newly recognized agents, later identified as adeno-

viruses, were reported independently by Kjellen (25) and Neva & Enders (26).

In December, 1954, Parrott *et al.* (27), reporting a hospital outbreak of febrile pharyngitis with conjunctivitis, subsequently named pharyngoconjunctival fever [Bell *et al.* (17)], suggested that the observed syndrome was a specific new clinical entity due to adenoviruses. In 1955, the role of these viruses in causing pharyngoconjunctival fever [Bell *et al.* (17)], epidemic keratoconjunctivitis [Jawetz *et al.* (28)], and follicular conjunctivitis [Huebner *et al.* (24); Ward *et al.* (29)] was reported. At the same time, Hilleman & Werner's (23) initial findings on adenovirus illnesses in military recruits were extended and confirmed by Hilleman *et al.* (30, 31), Ginsberg *et al.* (32), Berge *et al.* (33), and Rowe *et al.* (34), which clearly established the adenovirus etiology of acute respiratory disease (ARD) and certain viral pneumonias.

By the end of 1955, an experimental adenovirus vaccine was successfully tested in volunteers [Huebner *et al.* (35)]. In 1956, studies of adenovirus infections were reported from various parts of the world; these extended the current list of adenoviruses to 18 serotypes and further established them as causes of epidemic and endemic diseases, not only in military recruit populations, but in general populations as well (11). Also in 1956, experimental and commercially prepared vaccines [Hilleman *et al.* (36); Bell *et al.* (37)] were tested and found effective in preventing significant amounts of ARD and viral pneumonitis in military recruits. Thus, in three years, a new group of viruses was characterized and defined, much of its clinical importance determined, and a safe, effective commercial vaccine was made available for use. By 1957, an extensive literature, consisting of more than 150 scientific reports, had accumulated, including several symposia and reviews (1, 11, 38, 39, 40).

*Definition, characterization, and properties of adenoviruses.*—The adenoviruses comprise a group of viruses having common group-specific, complement-fixing (CF) antigens, and having the properties of ether resistance, cytopathogenicity for certain tissue cultures, and lack of pathogenicity for small laboratory animals [Enders *et al.* (2)].

The number of established serotypes in the adenovirus group now includes 18 types recovered from humans, four types from monkeys, and one type from chimpanzees [Rowe, Hartley & Huebner (41)]. In view of differences in the tissue-culture host range of the types recovered from different species, this laboratory has recommended division of the adenovirus group into types of human origin, expressed as numbered serotypes without a prefix, those of monkey origin prefixed with "M," and those of chimpanzee origin prefixed with "C" (41). Additional viruses known to possess the attributes of adenoviruses, in particular many of the simian viruses of Hull's cytopathogenic group No. 1 [Hull & Minner (42)], remain to be classified.

Evidence of serologic differences between strains of type 7 has been reported [Rowe, Hartley & Huebner (41); Pereira & Kelly (43)]. Re-

ciprocal neutralization tests with rabbit antiserums indicated that the majority of type 7 strains are immunologically distinguishable from the prototype strain, "Gomen," and that most of these are identical with one another; the subtype group has been referred to as "7a" (41).

There is much evidence to suggest that the adenoviruses of human origin could be classified into subgroups of biologically related types. On the basis of type of cytopathic and cytologic changes in HeLa cell cultures, the quantitative aspects of neutralization, tissue-culture host range, and epidemiologic behavior, types 1, 2, and 5 appear to make up a closely related group, as do types 3, 7, and possibly 4. However, until the extent of strain variation within the above types is determined and other serotypes are similarly studied, it is probably premature to formalize any subgrouping [Ginsberg (44)].

*Size.*—Hilleman, Tousimis & Werner (45) utilized a variety of biophysical procedures for estimation of the size of adenovirus type 4, including gradacol membrane filtration, pseudovelocity sedimentation, and electron microscopy of uranium-shadowed purified virus. The estimates obtained ranged from 80 to 120 mμ in diameter and were similar for virus from culture fluid or extracts of cells. On the other hand, electron micrographs of thin sections of infected HeLa cells indicate that the virus particles of all adenovirus types are 60 to 65 mμ in diameter [Harford *et al.* (46); Morgan *et al.* (47); Lagermalm *et al.* (48)]. Brandon *et al.* (49) observed concentrated adenovirus preparations by electron microscopy; in addition to two types of spherical particles, they observed 5-sided particles and long, slender filaments. Since the virus studied was prepared in monkey kidney cultures, it is possible that some of the structures observed were contaminating virus of monkey origin.

*Stability.*—In general, adenoviruses are very highly stable agents. They retain activity after storage for prolonged periods at 4°C., or after 30 min. exposure at room temperature to a pH range of approximately pH 2 to pH 10 [Rowe *et al.* (50); Ginsberg (51)]. Type 4 is relatively unstable on prolonged storage in an alkaline medium (51, 21). Infectivity is completely destroyed by heating at 56°C. for 30 min. Purified type 3 adenovirus is rapidly destroyed by chlorine, being inactivated at rates equivalent to *Escherichia coli* or *Salmonella typhosa* [Clarke, Stevenson & Kabler (52)]. Adenoviruses are also inactivated by formalin in concentrations of 1:400 to 1:4000, without loss of antigenicity [Huebner *et al.* (35); Hilleman *et al.* (36)].

*Host range.*—All known adenoviruses propagate and produce cytopathic changes in tissue cultures of HeLa or monkey kidney cells. The adenoviruses of human origin produce cytopathic changes in both of these tissues, but cannot be carried readily in serial passage in monkey kidney cells; conversely, the types recovered from monkeys propagate well in monkey kidney cells, but not in HeLa cells, although they produce severe cytopathic effects in first passage in the latter tissue. The chimpanzee type propagates in both

types of culture [Rowe, Hartley & Huebner (41)]. Strains of adenovirus types 1 through 6 have been adapted to growth in monkey kidney cultures, primarily by means of serial passage with concentrated inocula [Hartley, Huebner & Rowe (53)]. Several adenovirus types, particularly types 10, 12, 13, 15, 17, and 18 are poorly adapted to growth in tissue cultures, requiring several passages in HeLa or KB cells with large inocula before suitably active virus is obtained [Rowe & Huebner (21)].

The host range in tissue culture has been most thoroughly studied for types 1 to 4, and in particular for type 2 [Rowe *et al.* (22, 50)]. The latter virus was found to propagate in a wide variety of human and animal tissue cultures, including cells from rabbits, cotton rats, hamsters, and chick embryos. Types 3 and 4 were more restricted in host range; thus, types 1 and 2 propagated in rabbit trachea and cotton rat tissue cultures, while types 3 and 4 did not (50).

In addition to the human adenovirus—monkey kidney cell, and the monkey adenovirus—HeLa cell systems mentioned above, there are several other tissue culture systems in which adenoviruses produce marked cytopathic effects with little or no production of infectious virus; namely, types 3 and 4 in rabbit trachea (50), types 3, 4, and 7 in bovine tissue cultures [Warren & Cutchins (54)], and type 5 in rabbit fibroblasts [Pereira & Kelly (55)].

All adenovirus types have been found to be nonpathogenic for suckling and adult mice, and for adult rabbits. In addition, several human types have been inoculated into a wide variety of animals by a variety of routes without production of disease (23, 50). That certain species (guinea pigs, cotton rats, and hamsters) may be susceptible to some extent to human adenoviruses is suggested by the occurrence of antibody responses after a single inoculation of virus, in some cases after intranasal instillation (50, 56). Additional evidence of the susceptibility of guinea pigs is the occasional occurrence of CF antibody to adenoviruses in serums of uninoculated guinea pigs (39).

Despite the apparent insusceptibility of the rabbit to adenovirus infection, Pereira & Kelly (55) recovered type 5 from rabbit spleen removed as late as 55 days after intravenous inoculation; after the third day following inoculation, virus could be recovered only by growing the spleen in tissue culture and not by inoculating tissue suspensions into HeLa cell cultures.

*Quantitation.*—There are marked discrepancies in reports from different laboratories on the HeLa cell infectivity titers of adenoviruses, which doubtless have caused difficulty in comparison and interpretation of results. There are probably four chief reasons for these discrepancies: (*a*) differences in technique of virus titration; (*b*) differences in sensitivity of different HeLa cell systems for detection of virus [Grayston, Smith & McCarthy (57)]; (*c*) difference in yield of infectious virus in different HeLa cell systems [Ginsberg, Gold & Jordan (58)]; and, (*d*) difference between titers produced by different adenovirus types.

Of these differences, the first is almost certainly of greatest importance.

When adenoviruses are titrated in HeLa cells, a linear relationship is observed between the dilution of virus inoculated and the time of appearance of cytopathic changes [Rowe *et al.* (39); Roizman (59); Kjellen (60)]. Throughout most of the titration range, a tenfold dilution of virus results in a 1.2 to 2-day prolongation of the incubation period; near the titration endpoint the relationship is much more variable, and limiting dilutions may not produce cytopathic effects for as long as 20 to 28 days, or may be positive only on blind passage. Consequently, titration procedures using a short period of observation do not measure the actual amount of detectable infectivity, but give only a relative indication. Statements of ratios involving the absolute number of infectious units, such as the number absorbed or released per cell, when based on this type of titration, are misleading unless the ratio of the "relative" titer to the "actual" titer is known. On the other hand, short observation titrations appear to be ideally suited for comparison of quantity of virus in different preparations of the same adenovirus type, since they are terminated during the period when the regression line of dilution against incubation period is still in the linear phase. However, short observation titrations cannot be considered valid for comparison of titers of different adenovirus types until it is shown that the ratio of relative to actual titers is the same for each of the viruses compared. Because of the linearity of the dose-incubation period relation, it is also possible to quantitate adenoviruses by a single incubation-period determination, provided the test is controlled with a standardized preparation of the same virus type (59, 60). With this method of quantitation, it is important to use a dilution of $10^{-2}$ or higher, since "toxic" effects are produced by low dilutions of adenoviruses; these toxic effects can be produced by heat-inactivated preparations and produce a steeper slope of the regression line than infectious virus [Rowe *et al.* (21); Boyer, Leuchtenberger & Ginsberg (61); Pereira & Kelly (62)].

The sensitivity of HeLa cells to adenoviruses is influenced by the composition of the culture medium. Cultures maintained in media containing tryptose phosphate broth demonstrate a steeper slope of the "dilution-incubation period" regression line than cultures without the broth (58, 60). Also, inhibitors for adenoviruses are present in ox, calf, and horse serums [Gold & Ginsberg (63)], frequently used in tissue culture media.

*Serological tests.*—Neutralizing antibody response in humans infected with adenoviruses tends to be type-specific, but heterologous responses frequently occur; their occurrence is generally unpredictable, both as to the types showing rises and the degree of response [Huebner *et al.* (24); Grayston *et al.* (64); Roden, Pereira & Chaproniere (65); van der Veen & Kok (66)]. From the limited data available, one gains the impression that heterologous responses may be less frequent in children than in adults (21, 67). Grayston *et al.* (64) made extensive studies of the antibody responses to adenovirus types 1 to 10 in adults infected with types 3, 4, and 7; no clearcut pattern of heterologous responses was observed, and the occurrence of het-

erologous response was not correlated with the presence or absence of antibody to the heterologous type in the acute phase serum. Because of these cross rises, type-specific diagnoses by neutralization tests on paired serums, particularly from adults, are unreliable. Several outbreaks have been diagnosed retrospectively as to probable infecting type by means of neutralization tests on a number of paired serums [Ginsberg *et al.* (32); Cockburn, Rowe & Huebner (68)], but in one instance, subsequent virus-isolation studies showed that an epidemic initially considered to arise from type 4 [Forssel *et al.* (69)], was actually caused by type 7 [Oker-Blom *et al.* (70)].

The interpretation of serologic surveys is also made difficult by the frequency of heterologous neutralizing antibody responses; however, it is still not known whether heterologous antibody persists as long as homologous neutralizing antibody.

Neutralization tests with adenoviruses have generally been done by the conventional cytopathic endpoint technique, in which neutralization is defined as inhibition of cytopathic effects for an arbitrary time after inoculation or after the virus control cultures have shown effects (23, 50, 56).

Studies of the relation of the dose of virus employed to the neutralizing antibody titer obtained have been reported by Ginsberg (56), Roizman (59), and Kjellen (71). Ginsberg (56) showed that each fourfold decrease in quantity of virus resulted in a twofold increase in serum titer in tests with type 1 and 2, and a fourfold increase in titer with types 3 and 4; the results were the same with antiserums from humans, rabbits, or hamsters. In view of the dependence of sensitivity of test on the dose of virus employed and on the definition of neutralization, it is difficult to equate neutralization test results from different laboratories.

Several modifications of the neutralization test have been reported. Grayston *et al.* (72) evaluated a procedure in which culture tubes were planted with a mixture of HeLa cells, virus, and serum; the test was read for cytopathic changes at 24 to 48 hr. The results were considerably more reproducible by this method than with the standard neutralization procedure. Subsequently, Johnston, Grayston & Loosli (73) modified the test to use a colorimetric endpoint, using the yellow color of phenol-red indicator in acid medium as the indicator of virus activity. Lennette, Neff & Fox (74) also reported a colorimetric test for detection of adenovirus-neutralizing antibodies; by employing a smaller ratio of HeLa cells to virus, they obtained alkalinization of infected tubes, and consequently used the red color of phenol red as the index of virus.

Complement-fixing antibody responses to adenovirus infection generally show no type specificity, although occasional individuals show type-specific response. In our experience, the tendency to type specificity of CF antibody response has been most marked with type 8 infections. Two lines of evidence indicate that the CF antigens of different adenoviruses are not identical. Rowe *et al.* (50) reported that rabbit antiserums demonstrated a degree of type specificity in CF tests; Pereira (75) extended this finding,

and with carefully quantitated reagents was able to type strains of types 1 to 6 by complement fixation. Also, Friedman & DeBerry (76) found differences in heat stability at varying pH levels for the CF antigens of different adenovirus types.

Forsyth & Roizman (77) have reported concentration and purification of type 2 antigen by acid methanol-celite precipitation. No analyses of the chemical composition of the CF antigen have been reported; such a study would be of particular interest in view of the probable presence of deoxyribonucleic acid in the virus (78).

Direct hemagglutination with an adenovirus was first reported by Hull, Minner & Smith (79) with $SV_{15}$, a strain of type M-4. This virus agglutinates chicken, monkey, and human O erythrocytes. In recent experiments, Rosen (80) obtained evidence that the majority of adenoviruses produce hemagglutination of human, monkey, rat, or mouse erythrocytes in the usual test procedure, the cell susceptibility being different for different adenovirus types.

Friedman & Bennett (81) have described a serologic test for adenovirus infection using tannic acid-treated sheep erythrocytes which were sensitized with adenovirus; the addition of immune serum produced hemagglutination. Antibody responses detected by this test were less type-specific than neutralizing antibody responses, but were not so broadly type-nonspecific as CF antibody rises.

*Virus-cell interaction.*—Adenovirus infections of HeLa cells, particularly the patterns of virus multiplication and the biochemical and morphologic changes induced, have been studied in a number of laboratories. Adsorption of the virus is relatively slow, requiring 4 to 6 hr. [Roizman (59); Ginsberg (44, 82)]. Even with large inocula, only a relatively small proportion of cells in HeLa cell cultures is infected, as evidenced by the number of cells showing characteristic changes on cytologic (61) or electron microscopic (46) study. The length of the latent period before increase in titer of intracellular virus is 14 to 21 hr. (48, 82, 83), varying with virus type and with different stocks of HeLa cells (82). Increase in titer of extracellular virus occurs significantly later, and throughout the course of infection the titer of virus in the cells is higher than that in the culture medium (82). In contrast to morphologic evidence of viral replication in the nucleus (46, 47, 48), differential centrifugation studies indicate that the majority of infectious virus is in the cytoplasm [Denny, Boyer & Ginsberg (84)].

Levy *et al.* (85) reported a marked stimulation in uptake of isotopically labelled phosphate and glycine by HeLa cells infected with type 2 adenovirus as early as 2 hr. after inoculation; production of lactic acid increased at 8 hr. Increase of isotope uptake and lactic acid production continued after cytopathic changes were complete. Fisher & Ginsberg (86) found that increased acid production was accompanied by increased oxygen utilization, and that there was a concomitant accumulation of pyruvic, acetic, and α-ketoglutaric acids. These authors point out that it is not yet possible to

decide whether the increased acid production is an inherent part of viral synthesis or a nonspecific response to a particular type of cellular injury.

In HeLa cultures maintained in media with high serum concentration, a persistent infection may be obtained, virus multiplication occurring without production of cytopathic effects [Ginsberg (87, 88)].

Tissue culture cells infected with adenoviruses demonstrate a characteristic sequences of cytologic changes, particularly in the nucleus [Boyer *et al.* (61); Lepine *et al.* (89); Barski (90)]. Within 14 to 24 hr. after inoculation, multiple, small eosinophilic bodies appear, each surrounded by a clear zone; these bodies are generally Feulgen-negative. The inclusions increase in number, become more basophilic and Feulgen-positive, and the nucleus becomes enlarged and distorted. In the final stages, the inclusions coalesce into a deeply basophilic, strongly Feulgen-positive mass; the remainder of the nucleus either appears homogeneous and glassy, or is intensely vacuolated. A different sequence of changes is often seen with infection by types 3 or 4. A single granular or reticular mass appears in the nucleus, first stippled with Feulgen-positive granules and later becoming a basophilic and uniformly Feulgen-positive inclusion body. Types 3 and 4 also produce angular inclusion bodies having the appearance of crystals, chiefly in the clear zone of the nucleus (61). The cytologic changes produced by types 1, 2, 5, and 6 are identical, while a different pattern is seen with types 3, 4, and probably 7. Lepine *et al.* (89) considered monkey kidney cultures to be preferable for detailed study of the cytologic changes, and reported the changes produced by all adenovirus types tested to be essentially identical. However, Barski (90) noted differences between type 1 and 2 effects as compared with type 3 and 4 changes in monkey kidney cultures. Adenoviruses of simian origin produce cytologic changes identical to those produced by the human types in monkey kidney cultures [Chany & Rowe (91)].

Electron microscopic study of infected HeLa cells has likewise shown a characteristic pattern of nuclear changes [Harford *et al.* (46); Morgan *et al.* (47); Lagermalm *et al.* (48); Kjellen *et al.* (92)]. Areas of amorphous, osmiophilic material appear and, in or adjacent to these areas, viral particles are first seen. With increase in number, the virus particles are seen aligned in regular "crystalline" arrays, which have been interpreted as being cubic body-centered lattices [Low & Pinnock (93)]. The crystals eventually disintegrate, the nuclear membrane ruptures, and viral particles are liberated into the cytoplasm. By comparing adjacent sections of the same cell, Bloch *et al.* (78), using types 3, 4, and 7, determined that the initial amorphous material represented the Feulgen-negative stage of the inclusion, while the crystals were the Feulgen-positive inclusions.

The individual viral particles seen in the thin-section electron micrographs were of the order of 60 to 65 mμ in diameter and occasionally demonstrated internal structure consisting of a dense central body enclosed in a membrane.

Morgan *et al.* (94) observed a second type of intranuclear crystalline structure in HeLa cells infected with type 5 adenovirus. These bodies were composed of linearly arranged material spaced at 40 mμ, contained protein, and appeared to be devoid of nucleic acid. In view of the large size of these crystals, and the large proportion of cells in which they appeared, it is striking that they were not observed by Lagermalm *et al.* (48), who conducted extensive studies with another strain of type 5. A comparison of the biologic properties of the two strains would be of much interest.

Morgan, Rose & Moore (95) also reported a unique change occasionally observed in infected HeLa cells, consisting of aggregates of parallel, dense lamellae about 15 mμ in thickness; the lamellae appeared to be composed of poorly defined double membranes. Tubules composed of concentrically or spirally arranged membranes were also observed; it appears possible that the tubules represent the lamellar aggregates sectioned transversely.

*Oncolytic effects.*—Several attempts have been made to obtain oncolytic effects by inoculating adenoviruses into human tumors in patients or laboratory animals. Smith *et al.* (96) observed local necrosis in 26 of 40 cervical carcinomas inoculated directly or seeded by local intra-arterial injection with various adenoviruses; necrotic response appeared to be more frequent in patients without homotypic antibody in the preinoculation serum. In studies of 14 patients with various types of incurable malignant tumors, Southam, Hilleman & Werner (97) observed no oncolytic effects with type 4 adenovirus.

When adenoviruses were inoculated into HeLa cell tumors grown in the peritoneal cavity of x-irradiated cortisonized rats, no oncolytic effects were seen, and generally only small amounts of virus were recovered [Suskind *et al.* (98)]. In experiments with the H.Ep.-3 tumor in a similar system, Holzaepfel & Boutselis (99) obtained evidence of inhibition of tumor growth when type 3 virus was mixed with the tumor suspension prior to inoculation.

## Illnesses Due to Adenoviruses

*Acute Respiratory Disease (ARD).*—"Acute Respiratory Disease" is a term suggested in 1946 by the Commission on Acute Respiratory Disease for "a specific disease process due to a single or to a group of closely related agents" observed very commonly in military recruits (100). This clinical entity, described earlier as "febrile catarrh" by Stuart-Harris, Andrewes & Smith (101) was distinguished from afebrile "common colds" and primary atypical pneumonia, also frequently observed in military populations.

Ginsberg *et al.* (32), Berge *et al.* (33), Rowe *et al.* (34), Hilleman *et al.* (102), Dingle *et al.* (103), and Woolridge *et al.* (104) have shown the etiologic importance of adenoviruses types 3, 4, and 7 in producing a large proportion of acute, febrile respiratory illnesses in American military recruits. Balducci, Zaiman & Tyrrell (105), Andrews & McDonald (20), and van der Veen & Kok (66) reported similar observations in the British and the

Royal Netherlands Army, the latter reporting type 14 as a cause of ARD in recruits.

The clinical and epidemiologic features of ARD have been the subject of a number of reviews by Dingle *et al.* (103), Stuart-Harris (106), and Dascomb & Hilleman (107). Studies by Hilleman (16) demonstrated that as many as 90 per cent of cases of ARD in hospitalized military recruits could have been attributed to adenoviruses during certain epidemic periods. However, the impression that adenoviruses account for the majority of ARD cases in military recruits was not supported in studies at Fort Ord, California, by Berge *et al.* (33) and at Great Lakes Naval Training Station by Rowe *et al.* (34). They found during their studies that less than half of the cases classified as ARD could be attributed to adenovirus infections. In a recent appraisal of adenovirus as the cause of respiratory illness in military populations, Hilleman *et al.* (36) concluded that while adenoviruses cause a large proportion of the ARD in the military, a "sizeable proportion of undiagnosed respiratory illness remained to be studied among both recruits and seasoned military personnel."

The illnesses caused by adenoviruses in military recruits can be rather protean in nature [Dascomb & Hilleman (107)]. The most common clinical features appeared to be fever, pharyngitis, and nonproductive cough. However, such constitutional symptoms as malaise, myalgia, headache, and chills were also quite common. Pharyngitis and rhinitis were the most common upper respiratory signs and symptoms; however, conjunctivitis was occasionally observed in recruits infected with type 7, a few of the cases showing the characteristic pharyngoconjunctival fever syndrome. Otitis media and lymphadenopathy were also observed in a considerable proportion of cases. Tracheobronchitis with cough and chest pain occurred more often than not, whereas laryngitis, dyspnea, and frank pneumonitis occurred in a smaller proportion of cases, as did gastrointestinal signs and symptoms such as abdominal pain, nausea, and vomiting. The studies of Berge *et al.* (33) have considerable interest in that the diagnosis ARD was not employed as an all-inclusive diagnosis for the illnesses observed. Instead, five large categories of illness, based on anatomic localization of the major signs and symptoms, labeled respectively, tonsillitis, febrile common cold, nonstreptococcal pharyngitis, bronchitis, and primary atypical pneumonia, were employed. Adenoviruses were isolated from 38 per cent of the cases of tonsillitis, which was the lowest percentage observed, and from 48 per cent of febrile common colds, which was the highest percentage observed. This serves to point up not only the heterogeneity of the clinical manifestations produced by adenoviruses in military recruits, but it also suggests that some other or perhaps several other agents are still to be demonstrated as the cause of illnesses indistinguishable from those caused by adenoviruses.

*ARD in civilians.*—Surveys for neutralizing antibodies in serums of persons in several geographic locales, reveal an unexplained paucity of experience with the adenovirus types 4 and 7 in children (24, 67); however, such

antibodies appear during and following adolescence in both males and females, suggesting that infection with one or both of these types is not restricted to military populations (67). In one study, a majority of serums from females taken during pregnancy showed type 4 antibodies (24). In other studies (11, 64) 20 to 50 per cent of persons entering recruit training and 50 per cent of young reformatory inmates were shown to have antibodies to types 4 and 7.

Serologic studies of respiratory diseases in student populations show that two to eight per cent of illnesses similar to ARD may be attributable to adenoviruses (64, 108). An outbreak of influenzalike illness was observed by Borghans *et al.* (109) in a Netherlands industrial group. Only 20 per cent of the cases could be shown to be due to influenza virus, whereas 80 per cent of the noninfluenzal illnesses were shown to have a fourfold or greater rise in complement-fixation test to the common antigen of the adenovirus group. The specific serotype responsible for the adenovirus infections was not determined.

*Pharyngoconjunctival fever.*—Shortly after the discovery of adenoviruses, accidental infections with type 3 produced conjunctivitis in several laboratory workers (11). This led to subsequent investigations of outbreaks of febrile pharyngitis and conjunctivitis in the metropolitan area of Washington, D.C. (11). The first outbreak, described by Parrott *et al.* (27), occurred during February, 1954, in four children and four adults in the Clinical Center of the National Institutes of Health, Bethesda, Maryland. The illness was characterized by temperature ranging to 104°F., pharyngitis, rhinitis, cervical lymphadenopathy, and conjunctivitis. During the summer of 1954, Bell *et al.* (17) studied several outbreaks of acute respiratory illness with conjunctivitis linked with a primary epidemic in a summer day school. A triad of major clinical manifestations—fever, pharyngitis, and conjunctivitis—observed in all three outbreaks suggested that this was a highly specific and distinct clinical entity, and it was named pharyngoconjunctival fever. It now appears likely that this disease was recognized as a distinct entity on a number of occasions previously, perhaps first by Beal (110). A recent review of Japanese literature reveals that an illness similar to pharyngoconjunctival fever had been recognized in Japanese children since at least 1944; it was called "pseudomembranous conjunctivitis," and was believed to be a childhood manifestation of epidemic keratoconjunctivitis (111). It is likely that certain illnesses described by Derrick in 1943 (112) as "swimming bath conjunctivitis" and a Colorado outbreak of epidemic conjunctivitis studied in 1951 by Cockburn, which he called "Greeley Disease," also represented adenovirus infections (68, 113).

Subsequent to 1955, outbreaks of pharyngoconjunctival fever have been observed not only in the United States, but in Canada (19), England (114), Western Europe (69), and Japan (111). The original outbreaks observed by Parrott (27) and by Bell (17) were all due to type 3 adenovirus. Outbreaks of pharyngoconjunctival fever observed in an orphanage nursery

since 1955 have also, in nearly all instances, been due to type 3 (115). Outbreaks caused by types 7 and 14 have been reported in Canada (19) and in England (20). However, sporadic cases of pharyngoconjunctival fever have been studied in which types other than these were isolated; namely, types 1, 2, 5, and 6 (11, 19, 116). Most large epidemics of pharyngoconjunctival fever have been observed in the summer time (17, 69, 113, 114, 117 to 120); the illness can also be prevalent during the winter (27, 115, 121). In virtually all of the summer outbreaks, swimming either in pools or in lakes was the incriminating factor in the transmission of the disease.

*Acute febrile pharyngitis due to adenoviruses types 1, 2, 3, and 5.*—The ability of type 3 adenovirus to produce illness including nonbacterial pharyngitis was established in early studies (see previous section). On the basis of serologic surveys (11, 67) and their demonstration in adenoids and tonsils (50), types 1, 2, and 5 appear to be the most common of adenovirus infections. However, in the earlier studies, they were found only infrequently in relation to illness. Huebner *et al.* (24) reported the isolation of adenoviruses types 1 and 5 from throat swabs of two patients with acute pharyngitis; specific neutralizing antibody responses developed in both patients. In 1955, Ginsberg *et al.* (18) reported a family outbreak presumably due to type 2, Bell *et al.* (122) produced illnesses in volunteers with types 1 and 5, which resembled pharyngoconjunctival fever, and Roden *et al.* (65) produced febrile pharyngitis in volunteers with type 1. Rowe *et al.* (39), Jordan *et al.* (67), and Jawetz *et al.* (116) presented data which further suggested that types 1, 2, and 5 produced nonbacterial pharyngitis. Rowe *et al.* (39) reported 59 infections with types 1, 2, and 5, 46 (78 per cent) of which were temporally associated with febrile pharyngitis or febrile coryza. Subsequently, Bell *et al.* (115) has acquired convincing evidence concerning the etiologic roles of these types in producing acute febrile respiratory illnesses in infants. Epidemiologic analysis of acute febrile illnesses in 40 children with type 1 infections, 33 with type 2 infections, and 21 with type 5 infections, observed during a two-year period in an orphanage nursery (115), revealed significant associations of each virus with febrile illness. Of considerable interest was the apparent effect of prior infections with other adenoviruses on reducing the risk of subequent infection and of illness with types 1 and 2 (115). All children entering the orphanage were tested for serum-neutralizing antibodies against types 1, 2, 3, and 5, and then were observed regularly during an intensive longitudinal follow-up for the occurrence of illness and adenovirus infections. Prior homologous infection, as expected, greatly reduced subsequent infections for all types. Unexpectedly, prior infection with types 1, 3, or 5, which was not accompanied by detectable neutralizing antibody to type 2, reduced significantly the risk of subsequent infection and illness with the latter. Similarly, prior types 2, 3, and 5 infections reduced subsequent risk of experience with type 1, even though specific neutralizing antibodies to type 1 could not be demonstrated. Interestingly enough, prior type 1, 2, or 5 infections did not appear to influence

risk of subsequent infections with type 3 or illnesses due to such infection. Information concerning heterotypic antibody effects on type 5 was insufficient for analysis.

Types 1 and 2 represent the earliest and most common adenovirus infections of children; 70 to 80 per cent of infants in a densely populated urban area show evidence of infection by five years of age (11), and 40 to 60 per cent of suburban children show similar evidence of infection by the age of ten (67). Most type 3 and 5 infections tend to occur later than types 1 and 2 (11). Studies of acute, febrile, respiratory illnesses in general population groups, and in an orphanage nursery (11, 115) indicate that a total of 5 to 10 per cent of such illnesses occurring in children under the age of six may be attributable to infections with adenovirus types 1, 2, 3, and 5. Significantly fewer illnesses could be so explained in older age groups; for instance, in the general population, less than 0.5 per cent of persons over 17 with febrile respiratory illnesses showed evidence of adenovirus infections. It would appear, therefore, that infections of children represent by far the chief medical and public health problems produced by the adenoviruses (11).

*Acute conjunctivitis and epidemic keratoconjunctivitis (EKC).*—Adenovirus infections of the eye were recently reviewed in a Symposium on *Viral Keratoconjunctivitis* (38). The reports in 1954 by Huebner *et al.* (24) and Parrott *et al.* (27) of conjunctival infections caused by type 3, were quickly followed in 1955 by the demonstration of type 8 in a case of classic epidemic keratoconjunctivitis (EKC) [Jawetz *et al.* (28)]. Subsequent studies by Jawetz *et al.* (123, 124), Mitsui & Jawetz (125), Bietti & Bruna (126), and Vivell & Deibel (127) have established adenovirus type 8 as at least one of the causes of classical EKC in the United States, Canada, Switzerland, Germany, Austria, Italy, and Japan. Mitsui *et al.* (128) reproduced the typical illness in volunteers with adenovirus type 8.

In 1955, Fowle, Cockeram & Ormsby (129) reported the isolation of four strains of type 3 from patients with mild keratoconjunctivitis, an observation confirmed by Kimura *et al.* (130) in four of 27 cases. Thygeson (131) distinguished the corneal lesions observed in such cases from classical EKC on the following grounds: (*a*) that the infiltrates of EKC are usually grossly visible while those of pharyngoconjunctival fever are usually visible only with the slit lamp; (*b*) that the lesions of EKC are primarily subepithelial while those of pharyngoconjunctival fever are primarily epithelial; and (*c*) that when corneal changes occur in pharyngoconjunctival fever, they appear at the same time as the conjunctival changes, while in EKC they are delayed from seven to ten days. Another difference was the "greater likelihood of permanent corneal damage with EKC."

Although it is still too early to assess the total significance of adenoviruses in conjunctival and corneal disease, recent articles by Beale *et al.* (19) and Kimura *et al.* (130) may be of interest. Both studies represent surveys based on patients with follicular conjunctivitis seen in ophthalmologic clinics. The Canadian group isolated 32 strains of adenoviruses, types

2, 3, 7, and 9, from eye-washings of 55 patients with conjunctivitis (19). Kimura *et al.* (130) reports 13 isolations of types 2, 3, and 6 from 27 patients with conjunctivitis in California, and Murray *et al.* (132) isolated 12 strains of adenovirus from conjunctival scrapings from children in Saudi Arabia who had acute conjunctivitis. Types 3, 7, and 8 were included in the 12 strains; however, three new types now designated as serotypes 15, 16, and 17 were also recovered.

*Adenovirus pneumonia.*—Pneumonias due to adenovirus have been demonstrated both in adults and in children, and the few fatalities attributable to adenovirus infection have been associated with pneumonia in young infants. The role of adenoviruses in "atypical pneumonia without cold agglutinins" in military recruits was first demonstrated by Hilleman & Werner (23), and subsequently, by Berge *et al.* (33). Studies of primary atypical pneumonia "with cold agglutinins" in military recruits (32) and as observed in civilian studies (29), do not show serologic or other evidence of adenovirus etiology.

Chany *et al.* (15) summarized studies of 27 severe cases of pneumonia, including eight fatal cases, observed on the Pediatric Service of the St. Vincent de Paul Hospital in Paris, France, which were attributed to adenovirus infections. Four of the fatal cases studied appeared to be pathologically identical to a viral pneumonia described by Goodpasture and characterized by specific intranuclear inclusion bodies in cells of the pulmonary lesions (13, 14). The fatal cases in Paris were associated with type 7a infections (15); however, other fatal pneumonias have been associated with the isolation of serotypes 1, 2, 3, and 18 in the United States (24, 39), England (43), and Holland (43).

*Epidemiology of adenovirus illnesses.*—The epidemiology of adenovirus infections has been reviewed by Huebner *et al.* (11), Hilleman *et al.* (16), Bell (133), and Jordan (134) and will not be considered extensively here. Some of the important factors in the spread of adenoviruses are briefly considered as follows.

*Age and geographic factors.*—At least 8 of the 18 adenoviruses are known to be highly prevalent in one or another part of the world. The high prevalence of types 1, 2, 3, and 5 in infants and children, and 4 and 7 in military recruits in the United States, has been discussed in the previous section. In Japan (125) and in Saudi Arabia and Egypt, type 8 and perhaps other types appear to be quite prevalent in children (21). Type 14 has been reported in outbreaks of adenovirus illnesses in the Netherlands (66) and England (20).

*Sex.*—Sex does not appear to influence childhood infections with adenoviruses (17); however, serologic studies by Jordan *et al.* (67) showed significantly greater numbers of type 4 positives in adult males.

*Household versus school exposures.*—Unlike influenza and measles, the adenoviruses apparently do not spread readily in ordinary school environments, but like the Coxsackie, ECHO, and polioviruses, they tend to spread

readily in the home and in populations such as exist in orphanages, hospital nurseries, boarding schools, and summer camps. These environments, including military recruit centers, appear to provide the intimate type of exposure necessary for easy dissemination.

*Swimming.*—Swimming, both in pools and lakes, has been repeatedly associated with the spread of pharyngoconjunctival fever due to types 3 and 7a (69, 113, 114, 117, 118, 119). It has not been determined if this association arises from (*a*) contamination of the water with virus (which seems unlikely where large lakes are involved); (*b*) some other associated vehicle of dissemination; or (*c*) increased irritation of the conjunctiva, which has been suggested as a portal of entry of virus (122), by water, chlorine, or rubbing.

*Irritation of mucous membranes.*—The undoubted but unexplained role of swimming in producing adenovirus conjunctivitis and the probable role of corneal injury in the genesis of EKC [arc welding lamp exposure, foreign bodies in industry (135), and ophthalmologic instruments and solutions (136, 137)] suggested that local irritation of mucous membranes may provide a portal of entry for adenovirus. This concept was supported by studies in volunteers [Ward *et al.* (29); Bell *et al.* (122)], which showed that follicular conjunctivitis and pharyngoconjunctival fever could be reproduced regularly in susceptible persons by swabbing virus onto the conjunctival membrane; whereas such illnesses were seldom produced when virus was given by various other means, including intranasal instillation, by spraying, or even when the virus was dropped into the conjunctival sac.

*Season and climate.*—Adenovirus infections have been demonstrated in all months of the year, with perhaps greater prevalence of some serotypes during the cold months (16, 115). Epidemics of pharyngoconjunctival fever, however, appear to be most common in the summer. Serologic surveys have revealed evidence of adenovirus infections in nearly all parts of the world, including tropical areas (21).

*Incubation.*—The incubation period of pharyngoconjunctival fever due to type 3 appears to be five to seven days with a range of two to ten days (17). Patterns of adenovirus infection observed in volunteers inoculated with various serotypes (122) suggest that adenovirus illnesses caused by other serotypes may have similar incubation periods.

*Persistent infections.*—One of the outstanding features of infection with certain adenoviruses is persistence of the virus in pharyngeal lymphoid tissues. To demonstrate virus in adenoids and tonsils, it is necessary to cultivate the tissue *in vitro;* infected cultures undergo spontaneous cytopathic changes, and virus can be recovered in high titer from the culture fluids (22, 39, 43). Types 1, 2, and 5 appear to have a marked tendency to persist, but types 3, 4, 6, and 7a have also been recovered. The possibility has not been excluded that persistence depends on which virus infects an individual first, rather than on the property of the virus itself, since types 1 and 2 generally infect in infancy. The duration of persistence is not known, but can only be inferred from the high frequency of recovery of the same virus types from

both children and adults (39, 50). Pereira & Kelly (43) recovered type 7a from tissue cultures of the adenoids of a child 4 months after acute infection with the same type. These persistent infections presumably provide a reservoir of virus; in occasional children who are infected with types 1, 2, or 5, virus may be demonstrated intermittently in the throat or stool for months after the primary infection (11).

*Studies of adenoviruses in volunteers.*—Inoculations of volunteers with adenoviruses have been useful in delineating pharyngoconjunctival fever (29), acute febrile pharyngitis (65), follicular conjunctivitis (122), and epidemic keratoconjunctivitis (128), and in trials of an experimental vaccine (35). Perhaps of most practical importance, was the demonstration by Ward *et al.* (29) that objective illnesses could be regularly produced in volunteers by swabbing virus on the conjunctiva.

*Adenovirus vaccines.*—Efforts aimed at preventing adenovirus illnesses with vaccines have been remarkably successful. The first attempt, utilizing formalin-inactivated type 3 virus, grown in monkey kidney, provided volunteers with protection against an experimentally produced illness which was equivalent to the protection afforded by naturally acquired antibodies [Huebner *et al.* (35)]. This study was quickly followed by two successful field trials of similarly prepared vaccines in military recruits. Hilleman *et al.* (36) prepared an experimental vaccine containing types 4 and 7, which was successful in preventing ARD due to both types in vaccinated recruits. Significantly fewer total respiratory illnesses requiring hospitalization occurred in the vaccinated group (4.8 per cent) as compared with an unvaccinated control group (23.8 per cent).

A trivalent, commercially produced vaccine containing types 3, 4, and 7 was tested in Naval recruits during January to April, 1956 [Bell *et al.* (37)]. Significant protection against ARD and pneumonia due to type 4 adenovirus (the only type which was prevalent) was demonstrated. Serum neutralizing antibodies were produced against the serotypes included in each of the three vaccines.

The value of adenovirus vaccines in preventing respiratory and ocular illnesses in nonmilitary populations, some of which experience high illness rates due to adenoviruses, has not yet been determined. The results of studies currently in progress, on the value of vaccines in preventing types 1, 2, 3, and 5 illnesses in infant nursery groups (115), will be awaited with interest.

## New Myxoviruses

The properties of the myxovirus group have recently been defined by Andrewes, Bang, & Burnet (138). These properties are (*a*) hemagglutination of fowl red blood cells; (*b*) receptor-destroying enzyme associated with the virus; (*c*) destruction of erythrocyte receptors by RDE (receptor destroying enzyme of *Vibrio cholerae*); (*d*) removal of normal inhibitor from serum by RDE or periodate; (*e*) growth in the amniotic cavity of the hen's egg; (*f*) size of 80 to 150 mμ; (*g*) ether sensitivity; and (*h*) stability at −70°C.

Influenza A, B, and C viruses were the only myxoviruses known to cause human respiratory disease at the time the group was defined. Since then, four new members of this group have been recovered from persons with respiratory illness. Similar to the influenza viruses, one of the new agents, Sendai virus, propagates sufficiently well in eggs to permit primary isolation by amniotic inoculation (139, 140, 141). The other three viruses, CA and HA virus type 1 and type 2, propagate poorly in eggs, and tissue culture appears to be a more sensitive system for primary isolation (3, 8, 142).

The newly recovered viruses, along with Newcastle disease virus and mumps virus, appear to comprise a sub-group of the myxovirus group separable from influenza A, B, and C viruses. Each of these noninfluenza myxoviruses shares a common antigen or common antigens with at least one other virus of this subgroup. Mumps virus shares a common antigen or antigens with Newcastle disease virus (143, 144, 145), CA (3, 142) and Sendai (146, 147) virus, and Sendai virus is related to type 1 and 2 hemadsorption viruses (8, 148). It seems clear that much remains to be learned concerning the full serologic relationships among these viruses.

*Sendai (influenza D).*—This agent was first recovered in Japan by Kuroya, Ishida, & Shiratori (149) from an outbreak of pneumonitis in infants, by intranasal inoculation of mice with autopsy material. The validity of the original isolations has been questioned, since Kuroya *et al.* (149), Fukumi, Nishikawa, & Kitayama (150), Jo & Kitaoka (151), Sasahara, Hayashi & Kumagai (152), and Okuno (153) have recovered identical agents from normal mice. Subsequent studies, however, have supported the contention that this virus may be associated with human disease. Thus, pneumonitis was produced in a volunteer fed mouse passage virus by Kuroya *et al.* (149). A rise in serum antibody was demonstrated in this volunteer (149) and in another individual from whom the virus was isolated by Matsuzawa *et al.* (154). In addition, the virus was isolated in embryonated eggs by Ohashi (139), Yamada *et al.* (140), and Gerngross (141).

In the outbreak studied by Yamada *et al.* (140), an aseptic meningitis-like syndrome with pleocytosis was common. Five strains of virus were recovered in eggs from throat washings, cerebrospinal fluid, or blood.

During an epidemic of moderately severe influenza studied by Gerngross in Vladivostok (141), evidence was obtained that influenza A′ and Sendai virus were circulating simultaneously. A rise in hemagglutination-inhibition (H-I) antibody for Sendai virus was observed with 5 of the 11 paired sera studied by Gerngross (141), whereas at the height of the epidemic, only 26 per cent of the paired serum samples showed an H-I rise for influenza A′. The incidence of respiratory illness during this outbreak was more than twice the maximum for the preceding 12 years in Vladivostok, which was attributed by Gerngross to a lack of previous antigenic experience with Sendai virus. However, the evidence that the Vladivostok epidemic was caused predominantly by Sendai was inadequately supported, since only five virus strains were isolated and 11 paired serums studied.

Gorbunova *et al.* (155) have demonstrated H-I antibody in sera obtained

from individuals from Vladivostok eight months after the epidemic, whereas this antibody was absent from the serum of persons residing in Moscow, suggesting that Sendai virus did not spread throughout the entire USSR during 1956.

At the present time, the number of virus isolations is small and the pattern of illness attributable to Sendai virus has not been completely defined. However, the available evidence suggests that an influenzalike illness may occur in adults, while infection of infants and children may result in a severe and often fatal penumonitis or in an aseptic meningitis syndrome.

Sendai virus was recovered in eggs by Sasahara *et al.* (156) from swine with an influenzalike illness. Pulmonary edema, hemorrhage, and respiratory symptoms followed the administration of this agent to swine. Thus, it appears that Sendai virus has a broad host range with natural infection occurring in man, swine, and rodents.

The original investigation of Sendai virus by Kuroya *et al.* (149) established that the virus multiplied in the amniotic and allantoic cavity of the hen's egg, hemagglutinated chicken, guinea pig, human O, cow, and sheep erythrocytes, and was approximately 150 to 180 mμ in diameter. Hemolytic activity for fowl erythrocytes was demonstrated by Fukai & Suzuki (157). Jensen, Minuse & Ackermann (158) demonstrated that the erythrocyte receptors for the hemagglutinin were sensitive to RDE (receptor destroying enzyme of *Vibrio cholerae* filtrate) and confirmed the findings of Kuroya *et al.* (149) that Sendai virus was antigenically distinct from the other members of the myxovirus group—influenza A, B, and C, mumps, and Newcastle viruses. Jensen *et al.* (158) suggested that Sendai virus be classified as influenza D.

An antigenic relationship was demonstrated between Sendai virus and mumps virus by Gardner (146) and DeMeio & Walker (147), and between Sendai virus and the recently recovered HA viruses by Chanock *et al.* (8). Gardner (146), Bruce White, Gardner & Hope Simpson (159), and Sommerville (160) have reported Sendai virus infections in Great Britain. Some of the respiratory illness studied by these investigators was attributed to Sendai virus, since an antibody rise was demonstrated for this agent but not for mumps virus. However, in the absence of virus isolation, the serologic diagnoses of Sendai virus infection should be made with caution, in view of the antigenic relationship to the HA viruses and the possibility that other as yet unrecovered myxoviruses may share a common antigen.

*Croup-associated virus.*—During the winter of 1955, CA virus was isolated in monkey kidney tissue culture by Chanock (3, 142) from infants with croup in Cincinnati, and by Beale *et al.* (161) in HeLa and human amnion cultures from infants with the same illness in Toronto. Five of the 11 infants studied in Cincinnati developed antibody for CA virus. Fifteen infants were studied in Toronto, and virus was isolated from 10 of them. Chanock (3) showed that the strains isolated in Cincinnati were indistinguishable from the Toronto prototype strain. Recently, Cramblett (162) recovered CA virus from an infant with croup in Iowa City.

The total pattern of illness produced by CA virus is not known at present, and the contribution of this virus to the croup syndrome and to the overall respiratory disease picture awaits future studies. Recently, five viruses recovered during October, 1955, in Washington, D.C., from children with an influenzalike illness, were identified as CA virus by Vogel *et al.* (163). It is of some interest that these isolations were made at a time when CA virus was associated with croup in Cincinnati and Toronto, indicating the widespread dissemination of the virus at that time. The recovery of CA virus from individuals with an influenzalike illness, suggests that this agent produces a spectrum of respiratory illness with the severest manifestation being croup in young infants.

The properties of the virus, described by Chanock (3), place it in the myxovirus group. The "spongelike" syncytial cytopathogenic effect produced by this agent in monkey kidney and human amnion cultures appears to be distinctive and resembles only that seen with mumps virus, with which it shares a minor antigenic relationship (3).

*Hemadsorption viruses.*—When the newly described hemadsorption technique of Vogel & Shelokov (164) was used in conjunction with monkey kidney tissue culture, two new myxoviruses, tentatively designated as hemadsorption viruses, were isolated from children with respiratory illness by Chanock *et al.* (8). Type 1 hemadsorption virus was recovered from 35 children, 8 of whom (with pharyngitis, bronchiolitis, and pneumonia) were studied in hospitals, and 27 of whom were involved in an outbreak of febrile respiratory disease in a nursery. In a single day's test of all children in the nursery group, type 1 virus was significantly more prevalent among ill children, suggesting but not establishing an etiologic relationship. Type 2 hemadsorption virus was recovered from three children hospitalized with croup, and further studies are required to determine the etiologic importance of this agent. Preliminary studies involving 117 paired sera from children hospitalized during the winter of 1957 and 1958 with severe febrile respiratory disease, indicated that 28 per cent showed serologic evidence of concurrent infection with type 1 or 2 virus (148).

Monkey kidney tissue cultures infected with these viruses were capable of adsorbing guinea pig erythrocytes. During the initial passage in monkey kidney culture, these viruses produced a minimal cytopathogenic effect, and it is probable that many virus isolations would have been missed if the hemadsorption technique had not been used. However, hemadsorption is not a unique property of these viruses since Shelokov, Vogel & Chi (165) have shown that tissue cultures infected with other myxoviruses adsorb erythrocytes derived from various species.

Types 1 and 2 HA viruses were distinct from each other in H-I and neutralization tests with sera from immunized rabbits. Patients from whom type 2 virus was isolated developed CF and H-I antibody for type 1 virus, suggesting an antigenic relationship. However, individuals from whom type 1 virus was isolated failed in most instances to develop CF and H-I antibodies for type 2 virus. Type 2 virus was shown to be antigenically related

to, but different from, Sendai virus. Types 1 and 2 viruses were antigenically distinct from influenza A, B, and C, mumps, CA, and Newcastle disease viruses.

The properties of the hemadsorption viruses placed them in the myxovirus group. Chanock *et al.* (8) suggested that type 2 virus might well be considered as a variant of influenza D, American subtype, and type 1 virus may ultimately be classified as influenza E.

### Other Respiratory Viruses

*Respiratory syncytial virus.*—Morris, Blount, & Savage (4) studied an outbreak of coryza in a colony of chimpanzees held under observation for 3 to 24 weeks prior to the onset of illness. A virus, designated CCA (chimpanzee coryza agent), was recovered from one of the 14 affected chimpanzees, and the remaining 13 animals developed antibody to this virus during convalescence. When susceptible chimpanzees were inoculated intranasally with tissue culture CCA virus, coryza was observed after a three-day incubation period. During a study of infants with croup and pneumonia, Chanock, Roizman & Myers (5) recovered two viruses which were indistinguishable antigenically from CCA virus.

Syncytium formation was shown by Chanock *et al.* (5) to be the characteristic cytopathogenic effect in human liver and KB cultures. The CF antigen was found to be separable from the infectious particle by centrifugation. Sedimentation studies in sucrose solutions of varying densities indicated that the particle diameter was 90 to 130 mμ. These viruses did not propagate in eggs.

Using serologic techniques, Chanock & Finberg (166) demonstrated that infections of infants and small children in Baltimore were prevalent during the winter of 1956 to 1957. When paired serum specimens (average interval, 6 weeks) were collected from children without respiratory illness at the time of initial bleeding, antibody developed in seven per cent of individuals attending an outpatient clinic, and in 45 per cent of children who were hospitalized for noninfectious diseases. Additional evidence for widespread dissemination of virus was the finding that 50 per cent of children two years of age, and 77 per cent of children three years of age possessed neutralizing antibody.

The data obtained by Chanock & Finberg (166) suggested an etiologic association of this virus and pneumonia in small children. Thus, 9 of 13 children with pneumonia developed antibody, while the rate among 103 control children was ninefold less. Conclusions drawn from these data must be considered tentative in view of the small number of patients studied. Morris (167) has recently obtained evidence that this virus, or an antigenically related virus was associated with 13 per cent of noninfluenzal, nonadenovirus respiratory infections among United Nations troops in the Far East.

The name "chimpanzee coryza agent," appropriate at the time when virus was isolated from the chimpanzee outbreak, was thought by Chanock &

Finberg (166) to be too restrictive for these viruses in the light of more recent information on human infection. They suggested the designation "respiratory syncytial" virus, which indicates the association with respiratory tract and the predominant effect produced in tissue culture.

*Primary atypical pneumonia virus.*—The PAP virus isolated by Eaton, Meiklejohn & van Herick (168) in 1944, has been the subject of considerable controversy (169). Support for the existence of this virus and its association with primary atypical pneumonia was recently presented by Liu (170) and Liu, Eaton & Heyl (171). Using the indirect fluorescein-labeled antibody technique they isolated eight agents in eggs from specimens obtained from patients with primary atypical pneumonia. Four of the five patients tested showed a rise in antibody to these agents during convalescence. The agents were stated to have an antigenic relationship with the Mac strain of PAP virus isolated by Eaton *et al.* (168).

The strains were detected in infected chick embryos by the occurrence of fluorescence in the bronchiolar epithelium following the addition of fluorescein-labeled antibody. Thirteen-day chick embryos were found to be more sensitive to these viruses than 10- or 15-day eggs.

Future work with these agents will be awaited with great interest. Growth of PAP virus in tissue culture with the production of cytopathogenic changes or the demonstration of a CF antigen would facilitate progress by permitting epidemiologic studies of the overall contribution of these agents to respiratory disease.

*JH and 2060 viruses.*—Two similar, newly recognized viruses recovered from persons with mild respiratory illnesses were reported by Price (6) and by Pelon & Mogabgab (172) in 1956.

*JH virus.*—Price reported 20 strains of JH virus isolated in all seasons of the year from both adults and children (6). The illnesses associated with the viral isolations were characterized by malaise, coryza, and mild sore throat with a low-grade fever occurring in a minority of cases. About 20 per cent of persons over eight years of age were stated to have neutralizing antibodies in their serums. The virus was isolated in monkey kidney tissues, usually requiring a passage before typical cytopathogenic effects became visible. Rises in neutralizing antibodies were demonstrated in acute and convalescent serums of patients from whom virus was isolated. Epidemiologic evidence, sufficient to establish the JH virus as a cause of respiratory illnesses, was not presented in the original paper; however, in 1957, Price (173) reported that a vaccine prepared in monkey kidney cultures produced neutralizing antibodies and subsequently, during an outbreak of JH virus, prevented illness due to the virus. Twenty-six common coldlike illnesses were observed in a study group containing 50 vaccinated and 50 unvaccinated control children. Of the 26 illnesses, 23 occurred in controls and only three in the vaccinated group. Twenty of the 26 ill children responded with a fourfold or greater neutralizing antibody rise to JH virus. These remarkable reports remain to be supported by detailed clinical and epidemiologic

information. It is hoped that subsequent reports will fill in some of the gaps in the present information about the JH virus.

*2060 virus.*—This virus was isolated in 1954 from nasopharyngeal washings obtained from naval recruits with mild respiratory illnesses [Pelon *et al.* (7); Mogabgab & Pelon (174)]. Like JH virus, this agent grows slowly in monkey kidney cultures, often requiring a second passage before its characteristic cytopathogenic effects can be observed. The long (10- to 14-day) incubation period in tissue culture before cytopathogenic effects are seen also is similar to that observed with JH virus. Recruits with mild respiratory illness, from whom 2060 virus was isolated, responded with neutralizing antibodies in their convalescent serums, and serologic surveys revealed increasing prevalence of antibodies associated with increasing age of children.

The respiratory illnesses associated with 2060 infections were characterized by coryza with occasional mild sore throat or cough and temperature recordings of 100°F. or under, lasting for three to five days. Although evidence of 2060 infection was found more often than not in persons with such illnesses, Mogabgab & Pelon (174) concluded that the evidence supporting an etiologic relation to respiratory disease was circumstantial, and did not exclude the possibility of coincidental infection.

Attempts to produce useful complement-fixing or hemagglutinating antigens have been unsuccessful for both JH and 2060 viruses. Immunologic relationships between these viruses, if any, remain to be reported.

## LITERATURE CITED

1. Symposium, "Viruses in Search of Disease," *Ann. N. Y. Acad. Sci.*, **67**, (1957)
2. Enders, J. F., Bell, J. A., Dingle, J. H., Francis, T., Jr., Hilleman, M. R., Huebner, R. J., and Payne, A. M.-M., *Science*, **124**, 119 (1956)
3. Chanock, R. M., *J. Exptl. Med.*, **104**, 555 (1956)
4. Morris, J. A., Blount, R. E., Jr., and Savage, R. E., *Proc. Soc. Exptl. Biol. Med.*, **92**, 544 (1956)
5. Chanock, R. M., Roizman, B., and Myers, R., *Am. J. Hyg.*, **66**, 281 (1957)
6. Price, W. H., *Proc. Natl. Acad. Sci. U.S.*, **42**, 892 (1956)
7. Pelon, W., Mogabgab, W. J., Phillips, I. A., and Pierce, W. E., *Proc. Soc. Exptl. Biol. Med.*, **94**, 262 (1957)
8. Chanock, R. M., Parrott, R. H., Cook, K., Andrews, B. E., Bell, J. A., Reichelderfer, T., Kapikian, A. Z., Mastrota, F. M., and Huebner, R. J., *New Engl. J. Med.*, **258**, 207 (1958)
9. Committee on the Enteroviruses, National Foundation for Infantile Paralysis, *Am. J. Public Health*, **47**, 1556 (1957)
10. Huebner, R. J., *Symposium on Perspectives in Virology* (Rutgers University Press, New Brunswick, N.J., to be published)
11. Huebner, R. J., Bell, J. A., Rowe, W. P., *Cellular Biology, Nucleic Acids and Viruses*, **5**, 393 (Special Publications of the N.Y. Acad. Sci., 414 pp., 1957)
12. Rowe, W. P., and Huebner, R. J., *Am. J. Trop. Med. Hyg.*, **5**, 453 (1956)

13. Goodpasture, E. W., Auerbach, S. H., Swanson, H. S., and Cotter, E. F., *Am. J. Diseases Children*, **57**, 997 (1939)
14. Lelong, M., Lepine, P., Alison, F., Vinh, L. T., Satge, P., and Chany, C., *Arch. franç. pédiat.*, **13**, 1092 (1956)
15. Chany, C., Lepine, P. Lelong, M., Vinh, L. T., Satge, P., and Virat, J., *Am. J. Hyg.* (To be published)
16. Hilleman, M. R., *Ann. N.Y. Acad. Sci.*, **67**, 262 (1957)
17. Bell, J. A., Rowe, W. P., Engler, J. I., Parrott, R. H., and Huebner, R. J., *J. Am. Med. Assoc.*, **157**, 1083 (1955)
18. Ginsberg, H. S., Gold, E., Jordan, W. S., Jr., Katz, S., Badger, G. F., and Dingle, J. H., *Am. J. Public Health*, **45**, 915 (1955)
19. Beale, A. J., Doane, F., and Ormsby, H. L., *Am. J. Ophthalmol.*, **43**, 26 (1957)
20. Andrews, B. E., and McDonald, J. C., *Proc. Roy. Soc. Med.*, **50**, 753 (1957)
21. Rowe, W. P., and Huebner, R. J. (Unpublished data)
22. Rowe, W. P., Huebner, R. J., Gilmore, L. K., Parrott, R. H., and Ward, T. G., *Proc. Soc. Exptl. Biol. Med.*, **84**, 570 (1953)
23. Hilleman, M. R., and Werner, J. H., *Proc. Soc. Exptl. Biol. Med.*, **85**, 183 (1954)
24. Huebner, R. J., Rowe, W. P., Ward, T. G., Parrott, R. H., and Bell, J. A., *New Engl. J. Med.*, **251**, 1077 (1954)
25. Kjellen, L., *Arch. ges. Virusforsch.*, **6**, 45 (1955)
26. Neva, F. A., and Enders, J. F., *J. Immunol.*, **72**, 315 (1954)
27. Parrott, R. H., Rowe, W. P., Huebner, R. J., Bernton, H. W., and McCullough, N. M., *New Engl. J. Med.*, **251**, 1087 (1954)
28. Jawetz, E., Kimura, S. J., Hanna, L., Coleman, V. R., Thygeson, P., and Nicholas, A., *Am. J. Ophthalmol.*, **40**, 200 (1955)
29. Ward, T. G., Huebner, R. J., Rowe, W. P., Ryan, R. W., and Bell, J. A., *Science*, **122**, 1086 (1955)
30. Hilleman, M. R., Werner, J. H., Adair, C. V., and Dreisbach, A. R., *Am. J. Hyg.*, **61**, 163 (1955)
31. Hilleman, M. R., Werner, J. H., Dascomb, H. E., Butler, R. L., and Stewart, M. T., *Am. J. Hyg.*, **62**, 29 (1955)
32. Ginsberg, H. S., Badger, G. F., Dingle, J. H., Jordan, W. S., Jr., and Katz, S., *J. Clin. Invest.*, **34**, 820 (1955)
33. Berge, T. O., England, B., Mauris, C., Shuey, H. E., and Lennette, E. H., *Am. J. Hyg.*, **62**, 283 (1955)
34. Rowe, W. P., Seal, J. R., Huebner, R. J., Whiteside, J. E., Woolridge, R. L., and Turner, H. C., *Am. J. Hyg.*, **64**, 211 (1956)
35. Huebner, R. J., Bell, J. A., Rowe, W. P., Ward, T. G., Suskind, R. G., Hartley, J. W., and Paffenbarger, R. S., Jr., *J. Am. Med. Assoc.*, **159**, 986 (1955)
36. Hilleman, M. R., Stallones, R. A., Gauld, R. L., Warfield, M. S., and Anderson, S. A., *Proc. Soc. Exptl. Biol. Med.*, **92**, 377 (1956)
37. Bell, J. A., Hantover, M. J., Huebner, R. J., and Loosli, C. G., *J. Am. Med. Assoc.*, **161**, 1521 (1956)
38. Symposium on Viral Keratoconjunctivitis, *Am. J. Ophthalmol.*, **43**, 166 (1957)
39. Rowe, W. P., Huebner, R. J., and Bell, J. A., *Ann .N.Y. Acad. Sci.*, **67**, 255 (1957)
40. Ward, R., *J. Pediat.*, **49**, 480 (1956)
41. Rowe, W. P., Hartley, J. W., and Huebner, R. J., *Proc. Soc. Exptl. Biol. Med.*, **97**, 465 (1958)

42. Hull, R. N., and Minner, J. R., *Ann. N.Y. Acad Sci.*, **67**, 413 (1957)
43. Pereira, H. G., and Kelly, B., *Proc. Roy. Soc. Med.*, **50**, 755 (1957)
44. Ginsberg, H. S., *Ann. N.Y. Acad. Sci.*. **67**, 383 (1957)
45. Hilleman, M. R., Tousimis, A. J., and Werner, J. H., *Proc. Soc. Exptl. Biol. Med.*, **89**, 587 (1955)
46. Harford, C. G., Hamlin, A., Parker, E., and VanRavenswaay, T., *J. Exptl. Med.*, **104**, 443 (1956)
47. Morgan, C., Howe, C., Rose, H. M., and Moore, D. H., *J. Biophys. Biochem. Cytol.*, **2**, 351 (1956)
48. Lagermalm, G., Kjellen, L., Thorsson, K. G., and Svedmyr, A., *Arch. ges. Virusforsch.*, **7**, 221 (1957)
49. Brandon, F. B., Taylor, A. R., Patterson, M., and McLean, I. W., Jr., *Federation Proc.*, **16**, 407 (1957)
50. Rowe, W. P., Huebner, R. J., Hartley, J. W., Ward, T. G., and Parrott, R. H., *Am. J. Hyg.*, **61**, 197 (1955)
51. Ginsberg, H. S., *Proc. Soc. Exptl. Biol. Med.*, **93**, 48 (1956)
52. Clarke, N. A., Stevenson, R. E., and Kabler, P. W., *Am. J. Hyg.*, **64**, 314 (1956)
53. Hartley, J. W., Huebner, R. J., and Rowe, W. P., *Proc. Soc. Exptl. Biol. Med.*, **92**, 667 (1956)
54. Warren, J., and Cutchins, E. C., *Virology*, **4**, 297 (1957)
55. Pereira, H. G., and Kelly, B., *Nature*, **180**, 615 (1957)
56. Ginsberg, H. S., *J. Immunol.*, **77**, 271 (1956)
57. Grayston, J. T., Smith, M. E., and McCarthy, M. A., *Federation Proc.*, **16**, 416 (1957)
58. Ginsberg, H. S., Gold, E., and Jordan, W. S., Jr., *Proc. Soc. Exptl. Biol. Med.*, **89**, 66 (1955)
59. Roizman, B., *Quantitative Aspects of APC Virus—HeLa Cell Interaction*, (Doctoral thesis, Johns Hopkins Univ., Baltimore, Md., 1956)
60. Kjellen, L., *Arch. ges. Virusforsch.*, **7**, 110 (1956)
61. Boyer, G. S., Leuchtenberger, C., and Ginsberg, H. S., *J. Exptl. Med.*, **105**, 195 (1957)
62. Pereira, H. G., and Kelly, B., *J. Gen. Microbiol.*, **17**, 517 (1957)
63. Gold, E., and Ginsberg, H. S., *Federation Proc.*, **16**, 414 (1957)
64. Grayston, J. T., Loosli, C. G., Johnston, P. B., Smith, M. E., and Woolridge, R. L., *J. Infectious Diseases*, **99**, 199 (1956)
65. Roden, A. T., Pereira, H. G., and Chaproniere, D. M., *Lancet*, 592 (1956)
66. van der Veen, J., and Kok, G., *Am. J. Hyg.*, **65**, 119 (1957)
67. Jordan, W. S., Jr., Badger, G. F., Curtiss, C., Dingle, J. H., Ginsberg, H. S., and Gold, E., *Am. J. Hyg.*, **64**, 336 (1956)
68. Cockburn, T. A., Rowe, W. P., and Huebner, R. J., *Am. J. Hyg.*, **63**, 250 (1956)
69. Forssell, P., Lapinleimu, K., Strandstrom, H., and Oker-Blom, N., *Ann. Med. Exptl. et Biol. Fenniae (Helsinki)*, **35**, 287 (1956)
70. Oker-Blom, N., Wager, O., Strandstrom, H., Makela, P., and Jansson, E., *Ann. Med. Exptl. et Biol. Fenniae (Helsinki)*, **35**, 342 (1957)
71. Kjellen, L., *Arch. ges. Virusforsch.*, **7**, 307 (1957)
72. Grayston, J. T., Johnston, P. B., Smith, M. E., and Loosli, C. G., *J. Infectious Diseases*, **99**, 188 (1956)
73. Johnston, P. B., Grayston, J. T., and Loosli, C. G., *Proc. Soc. Exptl. Biol. Med.*, **94**, 338 (1957)

74. Lennette, E. H., Neff, B. J., and Fox, V. L., *Am. J. Hyg.*, **65**, 94 (1957)
75. Pereira, H. G., *J. Pathol. Bacteriol.*, **72**, 105 (1956)
76. Friedman, M., and DeBerry, P., *Bacteriol. Proc.* (*Soc. Am. Bacteriologists*), 72 (1957)
77. Forsyth, P. J., and Roizman, B., *Virology*, **5**, 393 (1958)
78. Bloch, D. P., Morgan, C., Godman, G. C., Howe, C., and Rose, H. M., *J. Biophys. Biochem. Cytol.*, **3**, 1 (1957)
79. Hull, R. N., Minner, J. R., and Smith, J. W., *Am. J. Hyg.*, **63**, 204 (1956)
80. Rosen, L., *Virology*, **5**, 574 (1958)
81. Friedman, M., and Bennett, C. R., *Proc. Soc. Exptl. Biol. Med.*, **94**, 712, (1957)
82. Ginsberg, H. S., *J. Exptl. Med.*, **107**, 133 (1958)
83. Liberman, M., and Friedman, M. *Bacteriol. Proc.* (*Soc. Am. Bacteriologists*), 72 (1957)
84. Denny, F. W., Jr., Boyer, G. S., and Ginsberg, H. S., *Federation Proc.*, **16**, 411 (1957)
85. Levy, H. B., Rowe, W. P., Snellbaker, L. F., and Hartley, J. W., *Proc. Soc. Exptl. Biol. Med.*, **96**, 732 (1957)
86. Fisher, T. N., and Ginsberg, H. S. *Proc. Soc. Exptl. Biol. Med.*, **95**, 47 (1957)
87. Ginsberg, H. S., and Boyer, G. S., *Federation Proc.*, **15**, 589 (1956)
88. Ginsberg, H. S., *Federation Proc.*, **16**, 414 (1957)
89. Lepine, P., Chany, C., Maurin, J., and Carre, M.-C., *Ann. inst. Pasteur*, **92**, 728 (1957)
90. Barski, G., *Ann. inst. Pasteur*, **91**, 614 (1956)
91. Chany, C., and Rowe, W. P. (Unpublished data)
92. Kjellen, L., Lagermalm, G., Svedmyr, A., and Thorsson, K. G., *Nature*, **175**, 505 (1955)
93. Low, B. W., and Pinnock, P. R., *J. Biophys. Biochem. Cytol.*, **2**, 483 (1956)
94. Morgan, C., Godman, G. C., Rose, H. M., Howe, C., and Huang, J. S., *J. Biophys. Biochem. Cytol.*, **3**, 505 (1957)
95. Morgan, C., Rose, H. M., and Moore, D. H., *Ann. N.Y. Acad. Sci.*, **68**, 302 (1957)
96. Smith, R. R., Huebner, R. J., Rowe, W. P., Schatten, W. E., and Thomas, L. R., *Cancer*, **9**, 1211 (1956)
97. Southam, C. M., Hilleman, M. R., and Werner, J. H., *J. Lab. Clin. Med.*, **47**, 573 (1956)
98. Suskind, R. G., Huebner, R. J., Rowe, W. P., and Love, R., *Proc. Soc. Exptl. Biol. Med.*, **94**, 309 (1957)
99. Holzaepfel, J. H., and Boutselis, J. G., *Cancer*, **10**, 577 (1957)
100. Commission on Acute Respiratory Diseases, *Am. J. Public Health*, **36**, 439 (1946)
101. Stuart-Harris, C. H., Andrewes, C. H., and Smith, W., *Med. Research Council* (*Brit.*) *Memr. Spec. Rep. Ser., 228* (1938)
102. Hilleman, M. R., Werner, J. H., Dascomb, H. E., and Butler, R. L., *Am. J. Public Health*, **45**, 203 (1955)
103. Dingle, J. H., Ginsberg, H. S., Badger, G. F., Jordan, W. S., Jr., and Katz, S., *Trans. Assoc. Am. Physicians*, **67**, 149 (1954)
104. Woolridge, R. L., Grayston, J. T., Whiteside, J. E., Loosli, C. G., Friedman, M., and Pierce, W. E., *J. Infectious Diseases*, **99**, 182 (1956)

105. Balducci, D., Zaiman, E., and Tyrrell, D. A. J., *Brit. J. Exptl. Pathol.*, **37**, 205 (1956)
106. Stuart-Harris, C. H., *Influenza*, 235 (Edward Arnold, Ltd., London, England, 1953)
107. Dascomb, H. E., and Hilleman, M. R., *Am. J. Med.*, **21**, 161 (1956)
108. Evans, A. S., and Morse, H., *J. Lab. Clin. Med.*, **46**, 812 (1955)
109. Borghans, J. G. A., Makstenieks, O., Versteeg, J., and Verlinde, J. D., *Ned. Tijdschr. Geneesk.*, **100**, 2110 (1956)
110. Beal, R., *Ann. oculist.* (Paris), (1907)
111. Tanaka, C., *Am. J. Ophthalmol.*, **43**, 46 (1957)
112. Derrick, E. H., *Med. J. Australia*, **2**, 334 (1943)
113. Cockburn, T. A., *Am. J. Ophthalmol.*, **36**, 1534 (1953)
114. Kendall, E. J. C., Riddle, R. W., Tuck, H. A., Rodan, K. S., Andrews, B. E., and McDonald, J. C., *Brit. Med. J.*, **2**, 131 (1957)
115. Bell, J. A., and Huebner, R. J. (To be published)
116. Jawetz, E., Hanna, L., Kimura, S. J., and Thygeson, P., *Arch. Internal Med.*, **98**, 71 (1956)
117. Sobel, G., Aronson, B., Aronson, S., and Walker., D., *J. Diseases Children*, **92**, 596 (1956)
118. Glander, R. von, Harnack, G. A., and Lippelt, H., *Deut. med. Wochschr.*, **81**, 1147 (1956)
119. Kjellen, L., Zetterberg, B., and Svedmyr, A., *Acta Paediat.*, **46**, 561 (1957)
120. Breckoff, E., *Deut. med. Wochschr.*, **81**, 1149 (1956)
121. Tyrrell, D. A. J., Balducci, D., and Zaiman, T. E., *Lancet*, **II**, 1326 (1956)
122. Bell, J. A., Ward, T. G., Huebner, R. J., Rowe, W. P., Suskind, R. G., and Paffenbarger, R. S., Jr., *Am. J. Public Health*, **46**, 1130 (1956)
123. Jawetz, E., Thygeson, P., Hanna, L., Nicholas, A., and Kimura, S., *Proc. Soc. Exptl. Biol. Med.*, **92**, 91 (1956)
124. Jawetz, E., Thygeson, P., Hanna, L., Nicholas, A., and Kimura, S. J., *Am. J. Ophthalmol.*, **43**, 79 (1957)
125. Mitsui, Y., and Jawetz, E., *Am. J. Ophthalmol.*, **43**, 91 (1957)
126. Bietti, G. B., and Bruna, F., *Am. J. Ophthalmol.*, **43**, 50 (1957)
127. Vivell, O., Zintz, R., and Deibel, R., *Deut. med. Wochschr.*, **82**, 100 (1957)
128. Mitsui, Y., Hanabusa, J., Minoda, R., and Ogata, S., *Am. J. Ophthalmol.*, **43**, 84 (1957)
129. Fowle, A. M. C., Cockeram, A., and Ormsby, H. L., *Am. J. Ophthalmol.*, **40**, 180 (1955)
130. Kimura, S. J., Hanna, L., Nicholas, A., Thygeson, P., and Jawetz, E., *Am. J. Ophthalmol.*, **43**, 14 (1957)
131. Thygeson, P., and Jawetz, E., *Am. J. Ophthalmol.*, **43**, 161 (1957)
132. Murray, E. S., Chang, R. S., Bell, S. D., Tarizzo, M. L., and Snyder, J. C., *Am. J. Ophthalmol.*, **43**, 32 (1957)
133. Bell, J. A., *Am. J. Ophthalmol.*, **43**, 36 (1957)
134. Jordan, W. S., Jr., *Ann. N.Y. Acad. Sci.*, **67**, 273 (1957)
135. Hogan, M. J., *Am. J. Ophthalmol.*, **43**, 41 (1957)
136. Cockburn, T. A., Nitowsky, R. T., and Cheever, F. S., *Am. J. Ophthalmol.*, **36**, 1367 (1953)
137. Thygeson, P., *Am. J. Ophthalmol.*, **43**, 98 (1957)
138. Andrewes, C. H., Bang, F. B., and Burnet, F. M., *Virology*, **1**, 176 (1955)

139. Ohashi, K., Kuroya, M., Ishida, N., and Shiratori, T., *Yokohama Med., Bull.*, **4**, 217 (1953)
140. Yamada, M., Sagae, K., Oshima, H., Arie, T., Ochi, M., Ichihashi, Y., and Nakao, T., *Virus (Osaka)*, **5**, 150 (1955)
141. Gerngross, O. G., *Problems in Virology*, **2**, 71 (1957)
142. Chanock, R. M., *Ann. N.Y. Acad. Sci.*, **67**, 287 (1957)
143. Kilham, L., Jungherr, E., and Lugenbuhl, R. J., *J. Immunol.*, **63**, 37 (1949)
144. Evans, A. S., *Am. J. Hyg.*, **60**, 204 (1954)
145. Bang, F. B., and Foard, M., *J. Immunol.*, **76**, 348 (1956)
146. Gardner, P. S., *Brit. Med. J.*, **I**, 1143 (1957)
147. DeMeio, J. L., and Walker, D. L., *J. Immunol.*, **78**, 465 (1957)
148. Chanock, R. M., Parrott, R. H., and Huebner, R. J. (Unpublished data)
149. Kuroya, M., Ishida, N., and Shiratori, T., *Yokohama Med. Bull.*, **4**, 217 (1953)
150. Fukumi, H., Nishikawa, F., and Kitayama, T., *Japan. J. Med. Sci. & Biol.*, **7**, 345 (1954)
151. Jo, K., and Kitaoba, M., *Virus (Osaka)*, **3**, 191 (1953)
152. Sasahara, J., Hayashi, S., and Kumagai, T., *Virus (Osaka)*, **5**, 19 (1955)
153. Okuno, Y. Cited by Fukai, K., and Suzuki, T., *Med. J. Osaka Univ.*, **6**, 1 (1955)
154. Matsuzawa, M., Shiratori, T., Ito, N., and Tanaka, N., *Virus (Osaka)*, **3**, 336 (1953)
155. Gorbunova, A. S., Gerngross, O. G., Gnorizova, V. M., and Bukrinskaia, A. G., *Problems in Virology*, **2**, 76 (1957)
156. Sasahara, J., Hayashi, S., Kumagai, T., Yamamoto, Y., Hirasawa, N., Munekata, K., Okaniwa, A., and Kato, K., *Virus (Osaka)*, **4**, 131 (1954)
157. Fukai, K., and Suzuki, T., *Med. J. Osaka Univ.*, **6**, 1 (1955)
158. Jensen, K. E., Minuse, E., and Ackermann, W. W., *J. Immunol.*, **75**, 71 (1955)
159. Bruce White, G. B., Gardner, P. S., and Hope Simpson, R. E., *Brit. Med. J.*, **I**, 381 (1957)
160. Sommerville, R. G., *Brit. Med. J.*, **I**, 1145 (1957)
161. Beale, A. J., McLeod, D. L., Stackiw, W., and Rhodes, A. J., *Brit. Med. J.*, **I**, 302 (1958)
162. Cramblett, H. (Personal communication)
163. Vogel, J., Shelokov, A., Bell, J. A., Davis, D., and Chanock, R. M. (To be published)
164. Vogel, J., and Shelokov, A., *Science*, **126**, 358 (1957)
165. Shelokov, A., Vogel, J., and Chi, L., *Proc. Soc. Exptl. Biol. Med.*, **97**, 802 (1958)
166. Chanock, R. M., and Finberg, L., *Am. J. Hyg.*, **66**, 291 (1957)
167. Morris, J. A. (Personal communication)
168. Eaton, M. D., Meiklejohn, G., and van Herick, W., *J. Exptl. Med.*, **79**, 649 (1944)
169. Horsfall, F. L., *Viral and Rickettsial Infections of Man*, 383 (J. B. Lippincott Company, Philadelphia, Penna., 719 pp., 1952)
170. Liu, C., *J. Exptl. Med.*, **106**, 455 (1957)
171. Liu, C., Eaton, M. D., and Heyl, J. T., *Bull. N.Y. Acad. Med.*, **32**, 168 (1956)
172. Pelon, W., Mogabgab, W. J., Phillips, I. A., and Pierce, W. E., *Bacteriol. Proc. (Soc. Am. Bacteriologists)*, 67 (1956)
173. Price, W. H., *Proc. Natl. Acad. Sci. U.S.*, **43**, 790 (1957)
174. Mogabgab, W. J., and Pelon, W., *Ann. N.Y. Acad. Sci.*, **67**, 403 (1957)

# THE USE OF BACTERIA GROWN *IN VIVO* FOR STUDIES ON THE BASIS OF THEIR PATHOGENICITY[1]

By H. Smith[2]
*Microbiological Research Establishment, Porton, Nr. Salisbury, Wiltshire, England*

The virulence and pathogenicity of microorganisms have been discussed in a number of excellent articles (1, 2, 3, 4, 5), in which the subject has been dealt with in a general fashion and the role of the host in the complex host-parasite relationship has been emphasized. This review, like a recent symposium on "The Mechanisms of Microbial Pathogenicity" (6), concentrates on the properties of the parasite rather than on those of the host. It is concerned with the nature of the products and metabolism of pathogenic organisms which, in contrast to nonpathogens, enables them to produce disease in a susceptible host; and it is especially concerned with a discussion of one particular method of recognizing and examining such compounds and metabolic processes.

With the notable exception of studies on the classical exotoxins, work on the chemical identification of bacterial products has been related more to antigenicity and behaviour in various serologic tests *in vitro*, than to the part they might play in a disease process. For this reason, many bacterial substances have been studied intensively although their contribution to the pathogenicity of the organisms is ill defined and in some cases appears to be negligible, e.g., the polysaccharide of *Bacillus anthracis* (7). In the context of this essay a bacterial product which will form a precipitate, fix complement, or perform any other serologic trick on the addition of an appropriate antiserum, is interesting but unimportant if it does not have any biologic property which materially contributes to the pathogenicity of the organism. Similarly, a particular bacterial product is not important just because it is an antigen, but only if its antigenicity contributes to active immunity against the disease. The general types of compounds and processes in which we are interested are discussed in the next section. The near synonymous terms "pathogenicity" and "virulence" are used so far as possible in the manner suggested by Miles (8), i.e., the former in respect to species and the latter in respect to strain within a species. The term "immunogenicity," means the ability to produce active immunity against the disease.

[1] The survey of the literature pertaining to this review was concluded in November, 1957.

[2] The author is indebted to Professor A. A. Miles, Dr. D. W. Henderson, Dr. J. Keppie, and Dr. T. W. Burrows for their critical reading of his manuscript.

## The Chemical Bases of Pathogenicity

A pathogen must first establish itself in the host and then produce disease. To do this, it must obviously have the inherent ability to grow and multiply in the nutritional conditions which exist in the host tissues while under microbial attack. These may not be merely physiologic conditions. Indeed, Dubos (5) has emphasized that conditions in tissues undergoing bacterial attack may be far from physiologic. The avirulence of some strains of a pathogenic species may well arise from the simple inability of the organism to grow and divide in this environment. Already a few examples are known of nutritionally exacting organisms which become virulent only when an essential nutrilite which is apparently lacking in the tissues of the host is supplied by injection (9, 10). It is unlikely that such an explanation for avirulence operates in many cases because of the relative nutritional completeness of blood and of tissue extracts. Avirulence is due rather to the nonproduction, for genetical or nutritional reasons, of a full complement of substances which, by acting in a more positive manner against the host, are responsible for the virulent properties. These substances can be grouped in either or both of two classes according to their presumed biologic function. First, there are the so-called aggressins or auxiliary pathogenic factors (8) which, although not necessarily toxic, combat the defence mechanisms of the host and allow the organisms to grow freely in the host tissues. Secondly, there are the compounds which cause the disease symptoms or death of the host. A single substance clearly could be in both classes. Various biologic tests for these two kinds of substances will be described later.

The magnitude of the harmful effects produced by individual bacteria *in vivo* varies from organism to organism as judged by the number of them which the host tolerates before finally succumbing to the disease. When this number is small, the harmful effect attributable to each organism is relatively large, e.g., in infections with *Clostridium tetani*. On the other hand, when the number is large as in anthrax, the harmful effect of the individual bacteria is relatively low and the role of the auxiliary pathogenic factors in the disease more obvious. It is the many organisms producing diseases of the latter type, rather than those characterized by a single powerful exotoxin, that offer the major problems in the study of pathogenicity. Here, virulence is due mainly to the possession of an armoury of products, all of which have to be present for maximum virulence. In some organisms, the activity of these virulence factors may be largely interdependent, and the absence of one component results in a striking loss of virulence although the other factors necessary for virulence are present. For example, the virulence of *Bacillus anthracis* is dependent on the production of two main factors —a capsule and the extracellular toxin (11). The probable reasons for the very low virulence of the Sterne and H.M. strains of *B. anthracis* is that the former lacks a capsule although it can produce toxin and the latter is capsu-

lated but lacks the ability to produce enough toxin (12). In other organisms, e.g., *Staphylococcus,* the activity of the virulence products may be more additive and the absence of one component may result in a relatively small reduction in virulence.

The classical method of comparing the properties of different virulent and avirulent strains of the same pathogenic species has contributed much to our knowledge of the compendium of properties which many organisms require for full virulence. The basis of the method is as follows; all virulent strains have a property or compound X which is not possessed by most avirulent strains and hence X is necessary for virulence. Very often this is the case. The speculations on the basis of virulence and immunogenicity of *B. anthracis,* made by Sterne (13) from a study of variants, have since largely been proved true. Recently, artificially produced mutants have been used in this manner (14), and the advent of serologic diffusion precipitin techniques has added precision to this approach (15), provided X is an antigen capable of producing precipitable antibody. It must be remembered, however, that the evidence thus obtained is only circumstantial. Until X has been shown to be produced by the organism during infection and to have some direct connection with virulence in biologic tests, the issue of its importance in the over-all virulence of the organism must be doubtful.

We turn now to the connection between the virulence factors of an organism and the production of immunity to the disease caused by the organism. It is almost certain that an immunogenic antigen must be in some form a factor involved in virulence, though all virulence factors need not be immunogenic, or even antigenic. In those organisms where the virulence factors appear to be additive in their action, significant active immunity may be obtained only by injecting a mixture of antigenic virulence factors. On the other hand, in those organisms where the virulence factors appear to be more interdependent, only one factor may need to be neutralised to effect protection against the disease. Thus, the toxin of *B. anthracis* is an important but not the only factor in virulence, yet it is the chief antigen for inducing immunity to the disease; the other important factor in virulence is capsular polyglutamic acid but neither it nor dead capsulated organisms containing it, possibly in combination with protein, will stimulate active immunity to anthrax (11, 16, 17, 18). Similarly, immunity to plague appears to have its basis in the production of antibodies to the auxiliary pathogenic factors of *Pasteurella pestis* rather than to the toxin which appears to take only a small part in the active protection mechanisms (19, 20). In some cases, the overt disease itself does not appear to stimulate a significant active immunity. This may be due to an inherent nonantigenicity of the bacterial products, but a comparative inaccessibility of the product to the antibody-forming apparatus (e.g., in skin infections) may be the cause.

In summary, therefore, we are interested in any facet of the pathogen's metabolism accompanying its growth in the host tissues, and in the compounds responsible for aggressive and toxic manifestations. As a corollary

to this, we are interested in the ability of any of the virulence factors to produce active protection against the disease realizing that many of them may not do so because, for some reason, they are poor antigens in the host under consideration.

## The *In Vivo* Environment for Producing All The Factors Responsible for Pathogenicity

The compounds responsible for the pathogenicity of an organism are usually proteins, carbohydrates, lipides and possibly nucleic acids, either existing free or present as mixed complexes, e.g., lipoproteins, mucopolysaccharides, etc. The same types of compounds are present in nonpathogenic species, and hence it is probable that the general metabolic processes whereby bacteria derive energy and synthesise the main structure of proteins, carbohydrates, etc., are similar for all organisms—pathogenic and nonpathogenic—and are not significantly altered by a change in conditions of bacterial growth. We are concerned, however, with the small peculiarities in molecular structure which make a compound toxic or aggressive. These peculiarities are certainly determined by the microbial genome but the production is phenotypically determined, especially by the nutritional conditions. Hence, though minimal media are useful in exploring the general processes of growth, reproduction, protein synthesis, etc., optimal media are of paramount importance when interest centres on the specific biologic activity of end products.

When a pathogenic organism grows in the tissues of a host and produces disease, we know—by definition—that all the compounds and processes involved in pathogenicity are being produced. Hence, the particular nutritional conditions of the host tissues during the infection and disease are fully adequate for the pathogen in regard to the formation of virulence factors. It must be emphasised here that the decisive nutritional conditions in the host tissues undergoing bacterial attack may be different from normal physiologic conditions and are continually changing (5). The pathogen may react in some way to these semipathologic conditions and to the mobilisation of the host defence mechanisms; in fact, some virulence factors may well be produced as a direct reaction to the defences of the host. It is, at present, impossible to reproduce *in vitro* even the physiologic conditions operative in the normal host, much less the conditions occurring during inflammation and tissue breakdown by bacterial products. Apart from the unknown influence of host metabolites and enzymes on the metabolism of the pathogen, the excellent gaseous exchange system of the mammalian body and its variation in inflammation would be difficult to reproduce. The peculiar nutritional and other environmental requirements of various pathogens which lead to their preferential attack on specific tissues in disease (e.g., ***Brucella abortus*** in the bovine placental cotyledon, *Corynebacterium diphtheriae* in the throat and tonsils, *Neisseria gonorrheae* in the urinogenital tract, etc.), are at present not understood and certainly not reproducible

*in vitro*. We shall see later with reference to the anthrax toxin that, paradoxically, the tissues of the infected host can protect a bacterial product from destruction by the parent bacterium. It is also possible that some pathogenic substances are large molecular compounds from the host which have been modified in some way by the pathogen. If this were so, it would be very difficult to produce the particular virulence factors in a synthetic medium without the addition of plasma or tissue components. In view of these considerations, it would be surprising if the complex metabolic activity of the pathogen *in vivo* was not different from that *in vitro*.

The successive loss of virulence and change in morphology during the artificial cultivation of recently isolated pathogens indicates the magnitude of the probable difference between organisms grown *in vivo* and *in vitro*. In this context, the S-R variation of many bacteria (21) is too well-known to need description. The fact that the virulence of an organism can frequently be increased by animal passage also implies that substances and processes connected with pathogenicity are produced *in vivo* (possibly as a result of mutation or selection of virulent types) to a greater extent than *in vitro*. The work of Braun (*22*) on the effect of animal products on the virulence of bacterial populations has emphasised the effect of the *in vivo* environment on the virulence of bacteria.

Another effect that may be the result of a difference between bacterial products found *in vivo* and *in vitro* is the efficacy of living, attenuated vaccines in cases where killed vaccines are useless, e.g., BCG vaccine in tuberculosis and S.19 vaccine for contagious abortion. These attenuated vaccines appear to grow to some extent in the tissues of the host and persist for relatively long periods (23, 24, 25); in this way, they may have an opportunity to produce essential antigens at the most effective site. The killed vaccines may be ineffective because antigens originally present in the cultures were destroyed by the sterilization process. However, the methods used for killing are usually relatively mild, and an alternative explanation is that the protective antigens are not present to any significant extent in the *in vitro* culture.

In recommending the use of organisms grown *in vivo* for studies on pathogenicity, there is no suggestion of the existence of any absolute difference between them and those grown *in vitro* of the kind postulated by Bail & Weil (26). Many virulence factors have indeed been recognised *in vitro*, e.g., the capsular polysaccharides of *Diplococcus pneumoniae* (*27*), the capsular polyglutamic acid of *B. anthracis* (28), and the envelope substance (Fraction I) of *P. pestis* (*29*, 30), and just as many virulence factors can be formed by avirulent organisms. But, in the diseased animal, a virulent organism is more likely to produce its whole armory of factors whereas, under conditions *in vitro*, there is a distinct possibility of one or more being missing. Nor is it beyond the realm of possibility that *in vitro*, compounds are formed which appear to be virulence factors but are never produced *in vivo*. When, however, the compounds responsible for patho-

genicity in the host are identified *in vivo,* their production in defined media is made much easier, e.g., the anthrax toxin first recognised *in vivo* was eventually produced *in vitro* (12).

We may, therefore, conclude that when there are gaps in our knowledge of the compounds and processes responsible for the pathogenicity of an organism, it will be profitable to study the organism and its products obtained from infected animals. This is certainly no new idea. The work of Bail & Weil (26) at the turn of the century concentrated the thoughts of bacteriologists on "animal" organisms but when it was found that a number of aggressins could be produced *in vitro* (31), interest in such organisms waned. In the last two decades, articles on virulence have contained numerous references to the special behaviour of pathogens in the *in vivo* environment. Unfortunately, there have been practically no accompanying experimental investigations. This is not, the author feels, because of any insuperable difficulty of obtaining and working with *in vivo*-grown organisms, but rather because of the attractive ease of working with organisms from artificial culture.

## The Isolation of Organisms Grown *In Vivo* and of Their Products

Until recently, there have been no studies either on the chemistry or on the metabolism of bacteria grown within the tissues of an infected mammalian host. Their extracellular products, present in exudates from infected animals, have received some attention. Bail & Weil (26) described the aggressins and immunising action of such exudates with particular reference to those from anthrax. Some 30 to 40 years later Cromartie *et al.* (32) prepared extracts of skin lesions from rabbits infected with *B. anthracis;* these extracts were chemically fractionated for an immunising antigen (33) and an inflammatory factor (34). Similar extracts from streptococcal (Group A) lesions in rabbits have also been investigated by Watson & Cromartie (35). In these studies, interest centred on extracellular products. The infecting organisms were not separated free from tissue constituents and examined closely.

Recently, Smith, Keppie & Stanley (36), upon re-examining the anthrax problem, developed a method for isolating pathogenic organisms from infected guinea pigs in sufficient quantity and of adequate purity to make possible a thorough examination of their chemistry and metabolism. Large quantities of host body fluids which contain products excreted by the pathogen were also obtained. The guinea pig was selected as the host for the infection as a compromise between two requirements. From the point of view of obtaining the maximum yield of products a large animal such as a sheep would have been best for the infection. However, it was desirable that the ultimate products of chemical extraction should be adequately tested for properties connected with virulence in an homologous system, and hence a species that could be tested in large numbers had to be used. Large guinea pigs (800 to 1000 gm.) were infected intraperitoneally and intrapulmonarily

with *B. anthracis;* the intrapulmonary injection insured a large volume of easily removable thoracic exudate. A few minutes after death, the thoracic and peritoneal exudates were collected and mixed. There were usually five volumes of exudate to one of plasma. By a three-step differential centrifugation at 0°, the body fluids and saline-washed bacteria were separated from blood cells. Dry *B. anthracis* (1.5 to 2.0 gm.) and 1.5 to 2.5 l. of body fluids containing the extracellular products of the organism were obtained from 100 guinea pigs. Bacteria, practically free from blood cells, were obtained by a process involving only a small over-all loss (10 to 13 per cent) of the total bacteria present in the harvested material.

This method is applicable to infections in which the causal organism is extracellular and can grow freely in the blood or in exudates. Thus, in a small scale trial (36) organisms of the following species were readily obtained from infected guinea pigs: *Streptococcus pyogenes, Diplococcus pneumoniae, Listerella monocytogenes,* and *Staphylococcus aureus.* Recently, Keppie, Smith & Cocking (37) have used the method for the routine collection of *P. pestis* grown *in vivo.* Large numbers of organisms were found in the thoracic and peritoneal exudates and both body cavities were washed thoroughly with buffered saline to provide the maximum numbers of organisms. Approximately 1 gm. of dried *P. pestis* organisms was obtained from 100 guinea pigs.

For special purposes, the route of infection can be varied. Thus, in later studies on *B. anthracis,* it was found that the extracellular toxin was concentrated in the plasma and not in the exudate (38). To collect toxic plasma, guinea pigs were infected intradermally, since this minimized the production of thoracic exudate and facilitated the collection of blood undiluted with exudate from the thoracic cavity after removing the heart and lungs.

In the isolation of organisms from intracellular diseases such as leprosy, tuberculosis, and brucellosis the organisms must be freed from their intimate relation with tissue cells.

Hanks (39) wished to measure the hydrogen transfer capacity of *Mycobacterium lepraemurium,* and since this organism is an obligate parasite he had to isolate it from *in vivo* sources. Infected testicular tissue was thoroughly homogenized at 0° in a 0.2*M* sucrose solution. After the removal of coarse tissue by slow centrifugation, the bacilli were separated from tissue components by two centrifugations through dense solutions—first, 0.3*M* (10.2 per cent w/v) sucrose and, second, 1.5*M* (11 per cent w/v) KCl—which tended to support and remove the tissue components. A microscopic count of a differentially stained film showed the washed bacterial deposit to contain less than 1 per cent of tissue debris. Gray (40) made further metabolism studies on *M. lepraemurium* separated by this method.

The main objection to the method of Hanks was the possible detrimental effect on bacterial surface components of the extremely concentrated electrolyte solution used in the separation. Recently, Segal & Bloch (41) have

described a method for separating *Mycobacterium tuberculosis* from the lungs of moribund mice, which overcomes this objection. The infected tissue was homogenised at high speed in isotonic sucrose (0.25*M*) solutions containing bovine albumin (0.25 per cent w/v), and the bacteria were separated from tissue components by differential centrifugation at 0°C. in this medium. The bacterial sediment was washed with saline containing albumin (0.25 per cent w/v) and was shown to be adequately free from tissue contamination by (*a*) microscopical examination and (*b*) by a comparison of the optical density, dry weight, and viable counts of suspensions with those of a similar suspension of *M. tuberculosis* grown *in vitro*. Relatively large quantities of organisms were obtained (4 mg. per infected mouse lungs), and they were used for a comparison of their properties with those of *in vitro* grown organisms, and for studies on virulence and immunity. Keppie, Fuller & Smith (42) have found a modification of the method of Segal and Bloch to be satisfactory for separating *Brucella abortus* from infected bovine cotyledon tissue.

Organisms separated by one of the methods described can be used directly for studies on their metabolism or extracted for chemical examination. Mild methods for extraction are essential and as far as possible all the bacterial substance must be examined. In addition, the extraction technique should, if possible, indicate the location of a particular product in the intact organism. If there is evidence of extensive lysis of the organism in the primary lesion or later in the disease, then, of course, the components inside the bacterial cell may be of importance in pathogenicity. When there is no demonstrable evidence of extensive lysis, e.g., in infections with *B. anthracis* or *P. pestis*, it seems that an aggressin or toxin must either be excreted or be present on the surface of the organism to have an effective role in pathogenicity. Indeed, an intracellular protein having a definite antiphagocytic action has been isolated from an avirulent phagocytosis-sensitive strain of *B. anthracis* (11).

For these reasons, therefore, particular attention should be paid to surface components of the pathogen. In this respect, it is important to make a thorough examination of the washings of the isolated bacteria for the presence of some easily removed surface component. The examination, at least for antigenic components, is greatly facilitated by the gel diffusion test. Using appropriate antisera, the washings of a certain number of organisms are compared against a lysate (or as complete an extract as possible) of the same number of washed organisms, and a volume of tissue fluid equal to that which originally contained the unwashed organisms. If the relative proportion of any antigen is larger in the washings than in the extract of whole organisms and the original tissue fluid, the antigen is probably a surface component. The preferential removal of capsular material, e.g., as in the work of Amies (43) with *P. pestis*, or the isolation of cell wall preparations (44) should always be attempted before an extract

of the whole organism is prepared. If this proves not to be possible, as in the case of *B. anthracis* (11), the subsequent localisation of any isolated component may prove difficult (11). However, phase contrast microscopy, as used by Tomscik (45) to detect precipitation patterns in capsules and in cell walls after the addition of appropriate antiserum, may be a valuable aid in deciding whether a compound is a component of the capsule or cell wall.

The extracellular products of the organisms can be obtained from infected body fluids or tissue extracts after filtration through thin, cellulose acetate filters having low adsorptive capacity. Chemical fractionation for biologically important compounds will prove difficult because the various bacterial products have to be separated from host components. In this context, it is important to confirm that the final product is of bacterial origin by making sure that it is specifically precipitated, or that its biological activity is specifically neutralised by homologous antiserum. If the final product is not antigenic, proof that it is a bacterial product must await its successful production *in vitro*.

There is one major difficulty inherent in the fractionation of these extracellular products. This is the possibility of antibody being produced in the donor animals before the collection of the bacteria and their products. Compounds subsequently isolated from such materials might be complexes of antigen and antibody in antigen excess. This is unlikely to happen in acute experimental infections such as those occurring after intraperitoneal and intrapulmonary injection of *B. anthracis* or *P. pestis* when the animal dies in 2 to 3 days. But in chronic infections such as brucellosis or tuberculosis, these antigen/antibody reactions may be a very real problem. A possible solution might be to grow the organism in foetuses or in eggs where no antibody is produced to complicate the issue.

Having obtained organisms grown *in vivo* and their products, we next turn to the tests we must apply to them in studies on pathogenicity. Before doing this, it is important to point out a defect of the methods described above for preparing organisms grown *in vivo* for studies on pathogenicity. All the methods of isolation are designed for organisms present in the host at a late stage of the disease for the simple reason that a maximum yield of bacteria is desired. It is conceivable, however, that bacteria vary in their possession of different virulence factors throughout the course of an infection, and for the full picture, organisms should be studied at an earlier stage of the disease. Miles *et al.* (46) have emphasised that the behaviour of the pathogen in the first few hours after the primary lodgement, at least in the skin, is of paramount importance in the eventual outcome of the disease. It is obvious, that any study of the pathogen *in vivo* and its repercussion on the host at this stage in the infection must rely on microtechniques. This type of study might be attempted by using some of Miles' skin infection methods (46), together with the new cytochemical staining tech-

niques which are now appearing for various compounds and reactions, e.g., the methods described recently by Holt (47) for the determination of the location and strength of enzymic action *in situ.*

### Biological Tests for Compounds Responsible for Pathogenicity

In work on the chemical basis of pathogenicity, we must study the metabolism of the pathogen for peculiarities due to the *in vivo* environment and examine any isolated compounds for their capacity as aggressins and as toxins. Metabolism studies may be made directly on the isolated organisms such as those described by Hanks (39) and Gray (40) for *M. lepraemurium* and Segal & Bloch (41) for *M. tuberculosis.* We shall see later that attempts can also be made to study some aspects of the metabolism of the pathogen while it is growing in the host.

To test for aggressins some knowledge is necessary of the methods whereby the host attempts to combat bacterial invasion, i.e., the mechanisms of so-called nonspecific resistance. Unfortunately, the individual mechanisms are, at present, incompletely understood and even less is known about the manner in which the different facets are integrated to form a collective defence system against the invading parasite (5, 46, 48, 49). In view of this, the most reliable test for an aggressin is its ability to enhance *in vivo* the virulence of the bacterial species from which it was derived. Here the aggressin has a chance of demonstrably affecting one or more of all the integrated defence mechanisms of the host, whereas any particular mechanisms selected for study of the aggressin *in vitro* may be irrelevant to defence *in vivo.* A quick and convenient method of making virulence enhancing tests is to inject intradermally. The size of the subsequent lesion is a measure of the degree of virulence enhancement (46).

However, a number of useful tests for aggressins can be made *in vitro;* they are based on isolated aspects of host defence mechanisms which appear to be important. It is reasonably certain that substances given out by the organisms or formed as a surface coat are aggressins if they will inhibit one or more of the following processes: (*a*) the phagocytosis of susceptible organisms by leucocytes; (*b*) the intracellular digestion of organisms should they be phagocytosed in a system such as that described by Mackaness (50); and (*c*) the killing of a susceptible organism by various sera and by white cell extracts, the two common sources of the numerous antimicrobial complexes (beta lysins, properdin, complement, basic peptides, phagocytin, etc.) which appear to play some part in host defence (5, 48, 49).

It is difficult to assess the importance of positive results from such isolated tests in the over-all pathogenicity of the organism. Thus, the relative occurrence and importance of these activities will vary among the pathogenic species and according to which host any one pathogen is invading. In deciding the importance of aggressin tests, it is most helpful to have the benefit of a study of the pathogen, and the reaction of the host to it, during the first few hours of infection. Studies of this type were carried out by Cro-

martie, Bloom & Watson (51) on infections of resistant and nonresistant animals with *B. anthracis*, and by Burrows & Bacon (52) on infections of mice with virulent and avirulent strains of *P. pestis*. Unfortunately, such studies are lacking in many diseases.

To follow chemical fractionation, it is convenient to assay aggressins by determining the minimal amount required to produce an arbitrarily chosen end point in standard biologic tests (17); samples from one fractionation can be examined in the same test as the initial material. When, however, an assay result is obtained for a purified preparation, an attempt should be made to assess the result in relationship to the concentration and position of the material in or outside the pathogen. For example, an extracted capsular component may show a lower activity per unit weight than an extracellular product in an antiphagocytic test, and yet be of equal or of greater importance in determining the resistance of the organisms to phagocytosis. In the capsule, it is concentrated where it is needed—between the phagocyte and the organism—and not dispersed throughout the surrounding medium.

Next we can consider how to test compounds thought to be responsible for the symptoms of the disease. If the disease is fatal, the material should be tested for lethality in the appropriate animal species. If, however, the disease is chronic or subacute (e.g., brucellosis, tuberculosis), the design of a relevant test for the harmful product is a difficult matter. A simple test for lethality in a species different from the host of the infection may be misleading. The best procedure is to decide from the general pathology of the disease (if this is known) what appear to be the important effects of the bacterial attack (e.g., *B. abortus* causes undulant fever and various sequelae in man and abortion in pregnant bovines) and to design realistic toxicity tests accordingly.

## SPECIFIC EXAMPLES OF THE USE OR POTENTIAL USE OF VARIOUS PATHOGENS GROWN *IN VIVO* FOR STUDIES ON PATHOGENICITY

We turn now to a description of the few examinations that have been made of organisms grown *in vivo*, commencing with some relatively extensive studies on *B. anthracis*.

### *Bacillus Anthracis*

The detailed literature on the pathology and immunochemistry of *B. anthracis* and its infections have been adequately reviewed elsewhere (53, 54, 55); only relevant aspects will be discussed here at appropriate points.

Studies on *B. anthracis* grown *in vitro* had shed little light on the compounds and processes responsible for its pathogenicity. Bail & Weil (26) had clearly demonstrated the aggressive activity of *B. anthracis*, but in almost half a century only scanty knowledge had accumulated about the chemical basis for it. Practically nothing was known about the killing power of *B. anthracis*. Neither the general nature of the lethal effect on the host

nor the products responsible for it were known. In particular, no lethal endo- or exotoxin had been found in artificial cultures of the organisms (53, 54, 56), and no study of the chemical basis of the lethal aspect of pathogenicity had been possible. Here, then, was a pathogen eminently suitable for study by the techniques discussed above.

During the war, 1939 to 1945, Cromartie, Bloom & Watson and their colleagues (32, 33, 34, 51) made attempts to isolate from the oedema fluid of rabbit skin lesions the extracellular compounds of *B. anthracis* responsible for some of the biologic activities that had been demonstrated in similar fluids by Bail & Weil (26). Their work has been reviewed by Treffers (57). Two factors were named—an "inflammatory factor" and a protective antigen. The inflammatory factor was separated and purified; the most purified preparation contained a large proportion of polyglutamic acid, but its electrophoresis diagram showed it to contain small amounts of other components. Partially purified preparations produced transient oedematous lesions in the skin of rabbits as did the crude extracts. Unfortunately, it is difficult to decide how much of the original "inflammatory activity" was separated into the partially purified preparations because no yields were quoted for the separations involved. The inflammatory factor neutralised the effect of the anthracidal substance from leucocytes; at least some of this activity was probably due to its content of polyglutamic acid since glutamyl peptide from *B. subtilis* had the same effect.

The supernatant obtained after co-precipitation of the inflammatory factor with calcium phosphate contained a protective antigen. It was not obtained in a purified condition but seemed to be protein and to migrate in the electrophoresis apparatus with the beta- and gamma-globulins. The inflammatory factor was not devoid of protective ability. One of two preparations separated by calcium phosphate was immunogenic (32), and two preparations protected animals when injected simultaneously with *B. anthracis* (34). The interpretation of these results in the light of later knowledge will be discussed below.

Recently, Smith, Keppie and their colleagues (7, 11, 12, 16, 17, 18, 36, 38, 58 to 64) have studied *B. anthracis* organisms and their products isolated from infected guinea pigs (*vide infra*) and have related their findings to the pathogenesis of *B. anthracis* in the host. In describing this work, the lethal aspect has been discussed before the aggressins because, in anthrax, the toxin is also an aggressin, and a definition of the toxin is necessary before an adequate discussion of its aggressive role can be made.

*The fatal syndrome in anthrax and the toxin responsible for it.*—The nature of the fatal syndrome and the recognition of the substance which caused it were investigated first. Hypotheses that, in anthrax, a massive bacteraemia led to blocked capillaries or to a deficiency of essential nutrients (e.g., $O_2$, glucose) were considered. To assess their importance, it was essential to know whether the massive bacteraemia was a necessary cause of death, since the literature was not clear on this point (55).

Guinea pigs were infected intradermally with virulent *B. anthracis*. On the third day and in the 12 hr. preceding death, the number of organisms in the blood rose from approximately $3 \times 10^5$ to $1 \times 10^9$ chains/ml. There was a remarkably constant relationship between the degree of bacteraemia and the subsequent survival time of an animal (60). Next, antibiotics were used to terminate the bacteraemia abruptly at progressive stages in its development, in order to detect the earliest stage at which damage to the host was sufficient to cause death. The administration of large quantities of streptomycin proved effective. Removal of the infection in this manner saved guinea pigs only when the bacteraemia was less than *ca.* $3 \times 10^6$ chains/ml. of blood, which occurred approximately 8 hr. before death if the normal course of the bacteraemia had proceeded. All treated animals with a bacteraemia greater than this critical value died 1 to 3 days later although free from infection. Hence, a bacteraemia is intimately associated with death from anthrax but, in fact, the death of the guinea pig is determined when the bacterial invasion is still only about 1/300th of its maximum.

These findings rendered untenable the earlier theories on cause of death, and emphasized the need for a renewed search for a toxic substance produced by *B. anthracis in vivo*. An insight into the probable biologic nature of this factor was provided by the identification of the hitherto unknown pathologic syndrome leading to death. This was achieved by making blood analyses, histologic examinations, and clinical observations on infected guinea pigs. The phase of the disease selected for these observations was the final 10 hr. of infection. The major pathologic changes were expected to occur during this time, because the studies on the bacteraemia described above had shown that the fate of infected animals was not decided until some 8 hr. before death.

The data that accumulated (61) from numerous determinations carried out on guinea pigs at various stages of this terminal phase of anthrax showed unequivocably the presence of reduced blood volume, reduced bleeding volume, reduced blood pressure, haemoconcentration, oedema, and haemorrhage, a fall in body temperature, interference in phosphate metabolism, disturbance of electrolyte balance, slugged blood and increased clotting time, interference with carbohydrate metabolism, acute renal failure, and loss of kidney alkaline phosphatase. These diverse signs fitted together completely to establish that severe secondary shock resulting from oligaemia played a major role in the death of guinea pigs from anthrax (61).

This examination of the chemical pathology of anthrax led to the recognition of the anthrax toxin. Since the guinea pigs died in secondary shock, an oedema-producing factor responsible for the oligaemia of the host should be found in the products of the pathogen and a search was accordingly made for this factor. There were already signs that it was present. A mixture of oedema fluid (5 parts) and plasma (1 part) produced transient oedema in the skin of guinea pigs (59), as had many similar preparations in the past (32, 54). The distribution and activity of this factor in the tissues of guinea

pigs dying of anthrax was re-examined. Particular attention was paid to the plasma and spleen, since an examination of the distribution of organisms had focussed attention on these as sites for toxin production (60) because they contained the most organisms. The oedema-producing activity was concentrated in the plasma (38). Intradermal injection of filtered heparinized plasma (0.2 ml.) from guinea pigs dying of anthrax produced an extensive area of oedema and congestion in the skin of normal guinea pigs. Skin tests on samples of plasma taken at intervals during the final bacteraemia showed that the tissue-damaging activity increased regularly with increase in number of the organisms in the blood (38). Oedema fluid, i.e., plasma which had escaped from the capillaries, had a negligible content. It was mainly oedema fluid that had been used by earlier workers (32, 59), and this explains their failure to recognise the toxin.

Undiluted plasma killed mice and guinea pigs when injected intravenously and intraperitoneally (38). The $LD_{50}$ of the toxic plasma for mice (22 gm.) and for guinea pigs (250 gm.) was approximately 0.4 ml. and 5 ml., respectively, by the intravenous route. The specificity of the oedema production and the lethal effect in mice and guinea pigs was established when these activities were neutralised by anthrax antisera prepared in the horse or in the guinea pig, using a live-spore vaccine (38). Guinea pigs which died after injection of the anthrax toxin were shown to have the same symptoms of severe secondary shock as had guinea pigs dying of infection. The toxin was extracellular; it could not be found in extracts of the organisms separated from the toxic plasma—extracts which were prepared by methods shown not to harm the toxin. It did not appear to be connected either with the capsule of the organism or with capsular polyglutamic acid for the following reasons: first, the antiserum from the horse which completely neutralised the toxin was prepared by injecting the Sterne uncapsulated strain of *B. anthracis;* second, polyglutamic acid isolated from *B. anthracis in vivo* (38) showing no evidence of heterogeneity, was nontoxic to mice and to guinea pigs and had no oedema-producing activity. The author is of the opinion that the inflammatory factor of Cromartie, Bloom & Watson (34), which was known to be impure, consisted of polyglutamic acid contaminated with a trace of the anthrax toxin which was responsible for the small skin reaction.

These findings opened the way to a chemical study of the basis of the lethality of *B. anthracis,* because the responsible product was available for chemical fractionation. The toxin consisted of two components which acted synergically in oedema production and lethality (18). One component (Factor I) was deposited quickly during ultracentrifugation, leaving the second component (Factor II) in the supernatant. The purification of these components proved difficult because of their extreme lability. They are stable at −20°C. but lose toxicity rapidly at 0°C. (62, 63). The most purified samples of Factor I consist of lipoprotein containing carbohydrate residues (63), and purified Factor II appears to be a protein (62). The mechanism

of their attack on the capillary walls to bring about the leakage which leads to fatal secondary shock is as yet unknown. They may be enzymes but as yet no substrate has been discovered.

*The aggressins of* B. anthracis.—Virulent *B. anthracis* resists phagocytosis and possibly extracellular lysis by an anthracidal substance present in the serum of some species and in extracts of leucocytes (55). The relative importance of these two defence mechanisms undoubtedly varies from species to species. The virulence-enhancing test noted above was supplemented by aggressin tests involving the inhibition of phagocytosis of *B. anthracis* by guinea pig phagocytes, and the inhibition of its lysis by the anthracidal substances in horse serum and in extracts of guinea pig leucocytes (11, 17, 64). The results of the application of these tests to fractions of toxic plasma and of organisms grown *in vivo* have done much to confirm and put in more concrete chemical terms what can be deduced from the literature (55) on the invasiveness of *B. anthracis*. At least two factors are involved—one acting extracellularly and the other connected with the capsule which is always present on virulent organisms.

The anthrax toxin plays a powerful role in invasiveness. Purified Factor I of the toxin has virulence-enhancing, antiphagocytic, and antianthracidal activity at low concentrations. It seems to have a direct harmful effect on phagocytes (64); a combination of Factors I and II has a powerful virulence-enhancing activity. The following fact emphasises the fundamental importance of the toxin as an aggressin. The toxin actively immunises animals against anthrax (18); the corresponding antibody or antibodies are effective against the organisms in the primary lesion (32, 51), which implies that the antigen must be an aggressin.

Polyglutamic acid was isolated as a sodium salt showing no evidence of heterogeneity from extracts of the organisms grown *in vivo* and from the tissue fluids (16). It had virulence-enhancing, antiphagocytic, and antianthracidal activity. The capsule of *B. anthracis* has been shown to contain polyglutamic acid (45) and hence is an important aggressin. It appears that the antiphagocytic activity of sodium polyglutamate is due to its antiopsonic action. (64).

In addition to capsular polyglutamic acid there was some evidence that a second aggressin existed in lipoprotein fractions isolated from extracts of organisms grown *in vivo* (11, 64). The importance of this substance in invasiveness has not been decided because there is so solid evidence that it is a surface component, although it does not appear to be produced by uncapsulated organisms (11, 64).

*The basis of immunity to anthrax.*—Pasteur (65) first vaccinated against anthrax using living, attenuated cultures. Dead bacteria and various bacterial extracts were ineffective. Bail & Weil (26) showed that sterile oedema fluid from infected animals immunised against anthrax. Using oedema fluid, Watson *et al.* (33) showed the protective activity to be associated with the beta and gamma globulins. Later, Smith & Gallop (17) isolated

a purified protective antigen (Fraction Y), taking advantage of its rapid sedimentation in the ultracentrifuge; it was a lipoprotein containing carbohydrate residues and had marked antiphagocytic properties. In *in vitro* cultures, a protective filtrate was first prepared in a serum medium (66, 67), then in a synthetic medium (68, 69), and later purified (70). Both *in vivo* and *in vitro* preparations were nontoxic.

The anthrax toxin and at least one of its labile components are immunising antigens (18, 38, 62, 63); they form the prime basis for active immunity to anthrax. Both the nontoxic immunogenic preparations are, however, intimately connected with the toxin and its components, since hyperimmune sera prepared against them will neutralise the oedema-producing and lethal effect of the anthrax toxin (17, 38). There is little doubt that Fraction Y isolated from oedema fluid was Factor I of the toxin which had been toxoided by the extraction procedures (17). The immunising filtrate from culture *in vitro* appears to contain components connected with both factors of the anthrax toxin since, like Factor II, it forms a synergic mixture with Factor I in tests for oedema-producing activity and lethality (38), and it has serologic precipitation lines common with both factors (63).

*Studies on the metabolism of* B. anthracis *growing* in vivo.—The metabolism of *B. anthracis in vivo* and its connection with virulence were studied (71, 72). The study was somewhat different to that of Segal & Bloch (41) on *M. tuberculosis* in which the organisms grown *in vivo* were harvested and then examined for metabolic activity *in vitro*. With *B. anthracis,* the organism growing in the blood stream during the final bacteraemia was studied *in situ*.

*B. anthracis* growing *in vivo* appeared to use relatively large quantities of glutamine, threonine, tryptophan, and glycine but not histidine, lysine, tyrosine, phenylalanine, methionine, or alanine. Next, an attempt was made to investigate the more specific role of various amino acids, purines, pyrimidines, and vitamins. *In vitro* the importance of each metabolite can be assessed by altering the composition of the environment so as to render it deficient in that compound. It was impossible to do this when dealing with metabolism of the pathogen in the tissues of a host. Hence, attempts were made to block the use of individual metabolites by injecting metabolite analogues, and to study the subsequent effects upon growth and the synthesis of toxin and capsular material. A number of analogues were screened for their effects during the terminal bacteraemic stage of anthrax in the guinea pig when growth and toxin production are remarkably constant. Analogues 8-azaguanine, 8-azaxanthine and, to a smaller extent, ethionine, α-amino *n*-butyric acid, and *p*-fluorophenylalanine inhibited the growth of *B. anthracis in vivo,* whereas 2-thiouracil and pyridine-3 sulphonic acid selectively inhibited toxin production. Inhibition of capsule formation was at no time demonstrable. The reversal of these inhibitions by mixture of the analogues with appropriate metabolites served as evidence that hypoxanthine, adenine, methionine, alanine, phenylalanine, and tryptophan are intimately

involved in the growth of *B. anthracis in vivo,* and pyrimidines and nicotinamide in toxin synthesis. It is interesting that of the amino acids which appeared to be of importance in the work with antimetabolites *in vivo*—namely, methionine, alanine, phenylalanine, and tryptophan—not one was indicated as important in a recent study (73) on the growth requirements of B. *anthracis in vitro.*

*The production of the toxin of* B. anthracis in vitro *(12).*—Toxin of *B. anthracis* was eventually obtained in a shaken culture (37°C.) of a virulent strain of *B. anthracis* in a mixture of tryptic meat broth and guinea pig plasma. Failure to detect the toxin in such cultures in the past arose from its very transient appearance at an unusually early stage of the culture. Under optimal conditions using an inoculum of $3 \times 10^7$ organisms, maximal toxin production occurred in 4 to 4.5 hr. and there was no toxin at 6 to 7 hr. The toxin was lethal and induced oedema; it immunised animals, consisted of two components and appeared in all tests to be identical with that produced *in vivo.* Extracts of organisms from 7-hr. cultures, in contrast to those from 2.5-hr. cultures, contained a potent toxin-destroying system. *In vivo* the toxin-destroying system either is not produced or is inactivated, thus allowing a continuous accumulation of the toxin as the bacterial content of the blood increases.

### *Mycobacterium Tuberculosis*

In spite of intensive work on tubercle bacilli grown *in vitro* and on tuberculosis, we have little knowledge of the substances or metabolic processes responsible for the intracellular multiplication of virulent organisms *in vivo* despite their importance in the invasiveness of this pathogen (74, 75). There is also disagreement (74, 75) about the substances responsible for the toxic and allergic symptoms of the disease. Finally, there are as yet no acceptable dead vaccines and recourse is still made to the use of a living vaccine of attenuated organisms, which are known to persist for some time in the tissues of the host (23). A study of *M. tuberculosis* grown *in vivo* might shed some light on these problems.

Sheehan & Whitwell (76) noticed a difference in fat staining between organisms of *in vitro* and *in vivo* origin, and the study of *M. tuberculosis* grown *in vivo* receives a powerful impetus from the recent work of Segal & Bloch (41, 77) which shows a clear-cut difference between bacilli grown *in vivo* and *in vitro.*

Tubercle bacilli isolated from infected mouse lungs had a lower hydrogen capacity than the same strain grown *in vitro;* the addition of various complex culture media had little or no stimulating effect on the endogenous respiration of the mouse-grown organisms, but they produced a very large oxygen uptake by the organisms from artificial culture. The two types of organisms were strikingly different in their response to various substrates. Of glucose, glycerol, lactic acid, sodium acetate, sodium pyruvate, sodium benzoate, benzaldehyde, sodium salicylate, *n*-heptanoic, octanoic, and oleic

acids, all of which elicited positive responses from the culture grown *in vitro,* only sodium salicylate and the latter three fatty acids had any effect on the mouse-grown organisms. A difference in surface structure between the two types of bacteria was suggested by the fact that organisms grown *in vivo* dispersed easily in saline or water, but those from artificial culture would not do so unless Tween was used. The bacteria grown *in vivo* were more virulent for mice than corresponding suspensions of bacilli of the same strain grown *in vitro.* Somewhat surprisingly, phenol-killed vaccines prepared from the mouse-grown organisms were less immunogenic than similar vaccines from organisms grown *in vitro.* Segal and Bloch suggested that this result might be due to the greater susceptibility of the organisms grown *in vivo* to phenol or that they contained less of a lipide adjuvant. It is possible, however, that the immunising antigen(s) produced by organisms grown *in vivo* might be predominantly an extracellular product or a surface component easily removed by washing in sucrose/albumin solutions. An active immunity test on sterile fluids from the infected lungs and the washings of the bacteria might have been revealing.

## Pathogenic Streptococci

Studies on pathogenic streptococci grown *in vitro* have revealed a complexity of different factors—O-haemolysin, S-haemolysin, erythrogenic toxin, fibrinolysin, ribonuclease, deoxyribonuclease, proteinase, leucocidin, hyaluronic acid, hyaluronidase, and type-specific M protein (78)—which may determine their pathogenic action and their role in rheumatism and heart disease. It seems probable that capsular hyaluronic acid and the M protein may contribute to resistance to phagocytosis, and the erythrogenic toxin cause the rash, but it is difficult to assess the relative importance of these and other factors in the over-all pathogenicity of streptococci *in vivo.* The current work of Watson and his colleagues (35, 79 to 83) on the products of streptococci grown *in vivo* may help in clarifying which of them indeed are produced in the environment of the host and may reveal important streptococcal products as yet unsuspected.

A significant feature connected with the generalized Shwartzman reaction was recognised as characterising the products of streptococci grown *in vivo.* Infection with streptococci Type A prepares a rabbit for the provocation of the generalised Shwartzman reaction by subsequent injection of toxins of Gram-negative organisms, whereas culture filtrates from *Streptococcus pyogenes* Type A or heat-killed organisms do not do so (84). Schwab, Watson & Cromartie (79) showed that rabbit skin lesions formed 24 hr. after intracutaneous injection of streptococcus Type A contained a substance which prepared normal rabbits for the provocation of a reaction very like the generalised Shwartzman reaction, involving myocardial necrosis, cortical necrosis of the kidney, and death. The reaction could be provoked not only by toxins of Gram-negative organisms but by filtrates rich in streptolysin O. This work with Streptococci grown *in vivo* had demonstrated that

a marked Shwartzman-like reaction could be obtained with preparing and provoking factors derived entirely from streptococcus A. The preparative factor was destroyed by heating, was of high molecular weight (82), and its specificity was established by demonstrating its neutralisation by a rabbit antiserum to the extract of skin lesions; the antiserum was type specific. Further information on its constitution is awaited. Thromboplastin (or necrosin) was also separated from the extracts of streptococcal lesions (81). This was not perhaps surprising since this substance is produced in general tissue injury (85). What is of primary importance is the product of the organism which leads to the liberation of thromboplastin.

### *Pasteurella Pestis*

The study of *P. pestis* grown ***in vivo*** has recently thrown light on some of the problems of pathogenesis and immunity in plague. Furthermore, it has been relatively easy with this pathogen to reproduce ***in vitro*** some facets of its pathogenicity and immunogenicity, which were first shown to be important using organisms grown ***in vivo***.

Burrows & Bacon (52, 86, 87) showed that virulent and avirulent strains of *P. pestis*, which were indistinguishable by any test ***in vitro***, could easily be recognised by their behaviour in the peritoneum of a mouse. The avirulent strain was phagocytosed progressively until all the bacteria disappeared. The virulent strain was also phagocytosed initially but within one-half hour some organisms had become resistant to phagocytosis; these multiplied and killed the host. This distinction between virulent and avirulent strains was then observed with organisms cultured under special conditions ***in vitro***. Recently Fukui *et al.* (88) have shown that *P. pestis* behaves in guinea pig lungs exactly as it did in Burrows' experiments. After an initial lag period, the virulent but not the avirulent cells became resistant to clearance by the lung macrophages. These experiments are similar to those formerly carried out by Wright (89) on pneumococci.

The ability of *P. pestis* to become resistant to phagocytosis in the absence of visible capsulation is associated with the presence of various antigens (87). These have been detected on gel diffusion plates but have not been isolated and their biological properties are unknown.

Recently, Keppie, Cocking & Smith (37, 90), using *P. pestis* isolated from infected guinea pigs, resolved the following anomaly in previous work on the pathogenicity of *P. pestis* and immunity to plague. Guinea pigs and mice are equally susceptible to a fatal infection with *P. pestis* and are both immunised by living attenuated vaccines, but the two species behave very differently in toxicity and immunity tests with fractions isolated from *P. pestis* grown *in vitro* (91). First, the so-called toxin of *P. pestis* kills mice but not guinea pigs (92, 93); either guinea pigs are killed by a toxin different from that which kills mice, or the toxin as previously isolated was not the native toxin that kills both species. Secondly, mice are effectively immunised by dead vaccines of whole organisms, whereas guinea pigs are not

solidly protected by dead vaccines, although they are by living, attenuated vaccines (94). Mice are immunized by the nontoxic envelope substance, Fraction I, obtained from saline extracts of acetone-dried *P. pestis*; but this material is only slightly immunogenic in guinea pigs (29, 30). The "residue antigen" left after extraction of acetone-dried organisms with saline, which constituted 70 to 75 per cent of the whole organism, is moderately immunogenic in guinea pigs but feebly so in mice (29, 30).

It now appears that the toxin and immunising preparations obtained from *P. pestis* grown *in vitro* are components of more complex products present in the organisms. Two preparations from *P. pestis* grown *in vivo* behave similarly in guinea pigs and in mice; one kills both species and the other immunises them (37, 90). The two preparations were obtained by ultrasonic disintegration of an aqueous suspension of *P. pestis* isolated from infected guinea pigs. The existence of any essentially extracellular toxin or immunising antigen had been ruled out by an examination of the infected plasma and exudates.

The soluble ultrasonic extract constituting *ca.* 70 per cent of the organisms killed both mice and guinea pigs. Furthermore, one lethal dose of toxin for a guinea pig could be obtained from an amount of *P. pestis* cells, less than that present in a guinea pig dying of plague. A reasonable explanation for the death of guinea pigs from plague had been obtained; the "mouse" toxin as described hitherto is not the native toxin or toxic complex of *P. pestic;* it is one component or a degraded form of it. The soluble extract had almost no protective effect for guinea pigs and was too toxic for adequate testing in mice.

The residue from ultrasonic treatment, constituting *ca.* 30 per cent of the organisms, almost certainly contained cell wall material. It dissolved almost completely after ultrasonic treatment in slightly alkaline buffers at pH 8.5 to 8.7. Either as a suspension in saline or as a solution, the material was nontoxic to mice and guinea pigs at high concentrations and actively immunised both species. It contained a large amount of lipide and although physically and chemically different from free Fraction I isolated from acetone dried organisms, it formed a line on Ouchterlony plates (15) which was common with that produced by free Fraction I. It also formed a line when examined for virulence antigens with antiplague serum absorbed with an avirulent organism as described by Burrows & Bacon (87). The relationship of the lines with one another and with the known virulence antigens is still under investigation. The complex was an aggressin because a solution of it prevented the phagocytosis of *P. pestis* by guinea pig polymorphonuclear cells.

Keppie, Cocking & Smith (90) suggested that *P. pestis* has a cell wall complex containing lipide, to which Fraction I is attached firmly near the cell wall and loosely in the body of the envelope. The same state of affairs probably exists with respect to the virulence antigens. This cell wall complex with the attached envelope can be regarded as the best antigenic material for the protection of all species. If it is split by autolysis, acetone drying,

and to a lesser degree by washing or treatment with ultrasonic waves, then the components lose some of their protective power. This loss may be more apparent in some species than in others as evidenced by the different protective power of free Fraction I for mice and guinea pigs.

The residue obtained after ultrasonic treatment of *P. pestis* might form the basis for an effective vaccine for man since it is nontoxic and immunises both mice and guinea pigs, two attributes not possessed by the dead vaccines previously used (94). The practicability of such a vaccine depended on the growth *in vitro* of organisms similar to those formed *in vivo*, and it now appears that this can be achieved. Ultrasonic extracts of virulent organisms grown under two conditions *in vitro* killed both mice and guinea pigs (38), and the residues remaining after ultrasonic extraction of a virulent strain (L 37) and an immunogenic avirulent strain (Ts) grown under certain conditions *in vitro* protected both species (90).

### *Brucella*

In spite of extensive research with organisms grown *in vitro*, the compounds and processes responsible for the all-important intracellular growth of this pathogenic species are unknown (95), and the most effective vaccination procedures demand live, attenuated vaccines (24, 25, 96). The study of the chemical basis of the pathogenicity of this species and of immunity to brucellosis using organisms grown *in vivo* should be a fruitful field of research. This view is strengthened by the work of Braun (22) on the effect of cellular products on the virulence of bacterial populations. Working mainly with the *Brucella* species, Braun showed that cellular products such as amino acids, furfurylamino purine, products of deoxyribonucleic acid (DNA) breakdown, etc., changed the virulence of cultures *in vitro*, and stated that "comparable selective effects probably occur *in vivo*." The author is unaware of any studies of the *Brucella* group *in vivo* despite the obvious implications of Braun's work and the following statement by Huddleson (95):

> One of the most challenging problems for the investigator in brucellosis of today and of tomorrow is the identification of the biochemical and biological factors which alter the symbiotic relationship between pathogenic brucella cells and the parasitized tissue cells to the extent that either a state of disease emerges or the bacteria are eliminated from the body. Further, the more we know about the biochemical and biological factors that influence the multiplication of micro-organisms *in vivo*, the better able we will be to envisage the processes by which the normal host destroys the invading parasite or permits co-existence without disease.

Recently Keppie, Fuller & Smith (42) have obtained sufficient *B. abortus* cells from infected bovine cotyledons for chemical work on some of the problems outlined above.

### *Pneumococcus*

Many studies of virulent pneumococci grown *in vitro* have clearly demonstrated that the capsular polysaccharides are responsible for their

resistance to phagocytosis (1, 27), but the basis of the killing power of pneumococci is still obscure. Studies of organisms grown *in vitro* have demonstrated a filterable haemolysin, but unlike those on pathogenic streptococcus these studies indicate that pneumococcus is not a particularly toxic organism (97). Studies with pneumococci grown *in vivo* may demonstrate the toxic processes responsible for death of the host.

## The Endotoxins and the Different Syndromes Caused by Gram-Negative Pathogens

Many different Gram-negative organisms produce endotoxins with similar chemical and physiologic properties (98). The author is of the opinion that this striking and important fact often leads to too simple generalisations concerning the basis of the pathogenicity of the Gram-negative organisms and to a disregard of the many differences that exist between them. Thus, the same type of toxin is produced by pathogenic and relatively nonpathogenic species (e.g., *Escherichia coli*). Different organisms grow in different locations in the host and give rise to different disease syndromes, e.g., the organism rapidly invades the blood stream in typhoid fever but not in bacillary dysentery (99, 100). The basis for these differences may be as important in pathogenicity or immunity as the possession of a common endotoxin. Different organisms will almost certainly have different nutritional requirements *in vivo*, and they may use different mechanisms of invasion. One wonders, if the Gram-negative organisms were examined closely, how many toxins different from their common endotoxins would become apparent, as already has been shown for *Shigella shigae* (101). In studies of the organisms in relation to their different disease syndromes rather than to their common endotoxin, the examination of organisms grown *in vivo* might be fruitful.

## Viruses

Although this essay deals only with bacteria, the substance of it would appear to allow one comment on current trends of research on animal viruses. Until the last decade, virologists were forced to study viruses isolated directly from an infected animal as has been recommended here for bacteria. Now, the rapid development of tissue culture techniques (102) is allowing the study of viruses in cells growing in relatively simple media. This will undoubtedly further fundamental studies on growth, multiplication, etc., under more amenable experimental conditions. Already, however, the cells from tissue culture are known to be quite different from the parent cells of the mammalian body (103 to 106). The possibility that any viruses that grow in these tissue culture cells are phenotypically altered by the change in growth conditions should be borne in mind in any studies involving complex viral products, e.g., studies on pathogenicity and on immunity.

In closing this essay, the author expresses the hope that if the topic is selected for review in the future, there will be available far more experi-

mental work on organisms grown *in vivo* than the scanty amount available at this time. It is hoped that the present review may help to stimulate such work.

## LITERATURE CITED


1. Dubos, R. J., *The Bacterial Cell,* 188 (Harvard University Press, Cambridge, Mass., 460 pp., 1945)
2. Raffel, S., *Stanford Med. Bull.,* **6,** 207 (1948)
3. Macleod, C. M., and Pappenheimer, A. M., Jr., *Bacterial and Mycotic Infections of Man,* 68 (J. B. Lippincott Co., Philadelphia, Pa., 785 pp., 1948)
4. Watson, D. W., and Brandly, C. A., *Ann. Rev. Microbiol.,* **3,** 195 (1949)
5. Dubos, R. J., *Biochemical Determinants of Microbial Disease* (Harvard University Press, Cambridge, Mass., 152 pp., 1954)
6. Howie, J. W., and O'Hea, A. J., *Symposium Soc. Gen. Microbiol., 5th Symp.* (1955)
7. Smith, H., and Zwartouw, H. T., *Biochem. J.,* **63,** 447 (1956)
8. Miles, A. A., *Symposium Soc. Gen. Microbiol., 5th Symp.,* 1 (1955)
9. Bacon, G. A., Burrows, T. W., and Yates, M., *Brit. J. Exptl. Pathol.,* **31,** 714 (1951)
10. Garber, E. D., Hackett, J., and Franklin, R., *Proc. Natl. Acad. Sci. U.S.,* **38,** 693 (1952)
11. Smith, H., Zwartouw, H. T., and Harris-Smith, P. W., *Brit. J. Exptl. Pathol.,* **37,** 361 (1956)
12. Harris-Smith, P. W., Smith, H., and Keppie, J., *J. Gen. Microbiol.,* (In press, 1958)
13. Sterne, M., *Onderstepoort J. of Vet. Sci. Animal Ind.,* **8,** 271 (1937)
14. Burrows, T. W., and Bacon, G. A., *Brit. J. Exptl. Pathol.,* **37,** 481 (1956)
15. Crumpton, M. J., and Davies, D. A. L., *Proc. Roy. Soc.* (*London*) [B]**145,** 109 (1956)
16. Zwartouw, H. T., and Smith, H., *Biochem. J.,* **63,** 437 (1956)
17. Smith, H., and Gallop, R. C., *Brit. J. Exptl. Pathol.,* **37,** 144 (1956)
18. Smith, H., Tempest, D. W., Stanley, J. L., Harris-Smith, P. W., and Gallop, R. C., *Brit. J. Exptl. Pathol.,* **37,** 263 (1956)
19. Jawetz, E., and Meyer, K. F., *J. Immunol.,* **49,** 1 (1944)
20. Pollitzer, R., *Plague,* 137 (Chronicle, World Health Organization, Monograph No. 22, Palais des Nations, Geneva, 698 pp., 1954)
21. Dubos, R. J., *The Bacterial Cell,* 200 (Harvard University Press, Cambridge, Mass., 460 pp., 1945)
22. Braun, W., *Ann. N. Y. Acad. Sci.,* **66,** 348 (1956)
23. Pierce, C. H., Dubos, R. J., and Schaefer, W. B., *Am. Rev. Tuberc. Pulmonary Diseases,* **74,** 683 (1956)
24. De Ropp, R. S., *J. Comp. Pathol. and Therap.,* **55,** 70 (1945)
25. Wilson Taylor, A., and MacDirmid, A., *Vet. Record,* **61,** 317 (1949)
26. Bail, O., and Weil, E., *Arch. Hyg.,* **73,** 218 (1911)
27. Felton, L. D., and Bailey, G. H., *J. Infectious Diseases,* **38,** 131 (1926)
28. Hanby, W. E., and Rydon, H. N., *Biochem. J.,* **40,** 297 (1946)
29. Baker, E. E., Somner, H., Foster, L. E., Meyer, E., and Meyer, K. F., *J. Immunol.,* **68,** 131 (1952)
30. Baker, E. E., Somner, H., Foster, L. E., Meyer, E., and Meyer, K. F., *Proc. Soc. Exptl. Biol. Med.,* **64,** 129 (1947)

31. Wilson, G. S., and Miles, A. A., *Topley and Wilson's Principles of Bacteriology and Immunity*, 1216 (Edward Arnold, Ltd., London, Engl., 2331 pp., 1955)
32. Cromartie, W. J., Watson, D. W., Bloom, W. L., and Heckly, R. J., *J. Infectious Diseases*, **80**, 14 (1947)
33. Watson, D. W., Cromartie, W. J., Bloom, W. L., Kegeles, G., and Heckly, R. J., *J. Infectious Diseases*, **80**, 28 (1947)
34. Watson, D. W., Cromartie, W. J., Bloom, W. L., Heckly, R. J., McGhee, W. J., and Weissman, N., *J. Infectious Diseases*, **80**, 121 (1947)
35. Watson, D. W., and Cromartie, W. J., *Rheumatic Fever*, 190 (Symposium, Thomas, L., Ed., University of Minnesota Press, Minneapolis, Minn., 349 pp., 1952)
36. Smith, H., Keppie, J., and Stanley, J. L., *Brit. J. Exptl. Pathol.*, **34**, 471 (1953)
37. Keppie, J., Smith, H., and Cocking, E. C., *Nature*, **180**, 1136 (1957)
38. Smith, H., Keppie, J., and Stanley, J. L., *Brit. J. Exptl. Pathol.*, **36**, 460 (1955)
39. Hanks, J. H., *J. Bacteriol.*, **62**, 521 (1951)
40. Gray, C. T., *J. Bacteriol.*, **64**, 305 (1952)
41. Segal, N., and Bloch, H., *J. Bacteriol.*, **72**, 132 (1956)
42. Keppie, J., Fuller, R., and Smith, H. (Unpublished data, 1956)
43. Amies, C., *Brit. J. Exptl. Pathol.*, **32**, 259 (1951)
44. Salton, M. R. J., *Symposium Soc. Gen. Microbiol., 6th Symp.*, 81 (1956)
45. Tomscik, J., *Symposium Soc. Gen. Microbiol., 6th Symp.*, 41 (1956)
46. Miles, A. A., Miles, E. M., and Burke, J., *Brit. J. Exptl. Pathol.*, **38**, 79 (1957)
47. Holt, S. J., *Biochem. J.*, **65**, 43P (1957)
48. Nungester, W. J., *Ann. Rev. Microbiol.*, **8**, 363 (1954)
49. Elberg, S. S., *Ann. Rev. Microbiol.*, **10**, 1 (1956)
50. Mackaness, G. B., Smith, N., and Wells, A. Q., *Am. Rev. Tuberc.* **69**, 479 (1954)
51. Cromartie, W. J., Bloom, W. L., and Watson, D. W., *J. Infectious Disease*, **80**, 1 (1947)
52. Burrows, T. W., and Bacon, G. A., *Brit. J. Exptl. Pathol.*, **35**, 134 (1954)
53. Eurich, E. W., and Hewlett, R. T., *System of Bacteriology*, **5**, 456 (Medical Research Council, London, Engl., 505 pp., 1930)
54. Soberheim, G., *Handbuch der Pathogenen Mikro-organismen*, **3**, part 2, 1041, (Gustav Fischer, Verlag, Jena, Germany, 1826 pp., 1931)
55. Smith, H., and Keppie, J., *Symposium Soc. Gen. Microbiol., 5th Symp.*, 126 (1955)
56. King, H. K., and Stein, J. H., *J. Gen. Microbiol.*, **4**, 48 (1950)
57. Treffers, H. P., *Ann. Rev. Microbiol.*, **1**, 263 (1947)
58. Smith, H., Keppie, J., and Stanley, J. L., *Brit. J. Exptl. Pathol.*, **34**, 477 (1953)
59. Keppie, J., Smith, H., and Harris-Smith, P. W., *Brit. J. Exptl. Pathol.*, **34**, 486 (1953)
60. Keppie, J., Smith, H., and Harris-Smith, P. W., *Brit. J. Exptl. Pathol.*, **36**, 315 (1955)
61. Smith, H., Keppie, J., Stanley, J. L., Harris-Smith, P. W., *Brit. J. Exptl. Pathol.*, **36**, 323 (1955)
62. Sargeant, K., and Smith, H. (Unpublished data, 1957)

63. Stanley, J. L., and Smith, H. (Unpublished data, 1956)
64. Keppie, J., Smith, H., Harris-Smith, P. W., and Gallop, R. C., *Brit. J. Exptl. Pathol.* (In press, 1958)
65. Pasteur, L., *Compt. rend.*, **92,** 429 (1881)
66. Gladstone, G. P., *Brit. J. Exptl. Pathol.*, **27,** 394 (1946)
67. Gladstone, G. P., *Brit. J. Exptl. Pathol.*, **29,** 379 (1948)
68. Wright, G. C., Hedberg, M. A., and Slein, J. B., *J. Immunol.*, **72,** 263 (1954)
69. Belton, F. C., and Strange, R. E., *Brit. J. Exptl. Pathol.*, **35,** 144 (1954)
70. Strange, R. E., and Belton, F. C., *Brit. J. Exptl. Pathol.*, **35,** 153 (1954)
71. Smith, H., and Tempest, D. W., *J. Gen. Microbiol.*, **17,** 731 (1957)
72. Tempest, D. W., and Smith, H., *J. Gen. Microbiol.*, **17,** 739 (1957)
73. Oikawa, T., *Nippon Saikingaku Zasshi,* **11,** 1099 (1956)
74. Dubos, R. J., *Biochemical Determinants of Microbial Disease,* Chap. 4 (Harvard University Press, Cambridge, Mass., 152 pp., 1954)
75. Dubos, R. J., *Symposium Soc. Gen. Microbiol., 5th Symp.*, 103 (1955)
76. Sheehan, H. L., and Whitwell, F., *J. Pathol. Bacteriol.*, **61,** 269 (1949)
77. Segal, W., and Bloch, H., *Am. Rev. Tuberc., Pulmonary Diseases,* **75,** 495 (1957)
78. Wilson, G.S., and Miles, A. A., *Topley and Wilsons' Principles of Bacteriology and Immunity,* 662 (Edward Arnold, Ltd., London, Engl., 2331 pp., 1955)
79. Schwab, J. H., Watson, D. W., and Cromartie, W. J., *Proc. Soc. Exptl. Biol. Med.*, **82,** 754 (1953)
80. Watson, D. W., *Streptococcal Infections,* 92 (Columbia University Press, New York, N.Y., 218 pp., 1954)
81. Schwab, J. H., and Watson, D. W., *J. Infectious Diseases,* **95,** 267 (1954)
82. Schwab, J. H., Watson, D. W., and Cromartie, W. J., *J. Infectious Diseases,* **96,** 14 (1955)
83. Watson, D. W., *Federation Proc.*, **16,** 437 (1957)
84. Thomas, L., Denny, F. W., and Floyd, J., *J. Exptl. Med.*, **97,** 751 (1953)
85. Stoner, H. B., and Green, H. N., *Brit. J. Exptl. Pathol.*, **32,** 183 (1951)
86. Burrows, T. W., and Bacon, G. A., *Brit. J. Exptl. Pathol.*, **37,** 286 (1956)
87. Burrows, T. W., and Bacon, G. A., *Brit. J. Exptl. Pathol.*, **37,** 481 (1956)
88. Fukui, G. M., Lawton, W. D., Janssen, W. A., and Surgalla, M. J., *J. Infectious Diseases,* **100,** 103 (1957)
89. Wright, H. D., *J. Pathol. Bacteriol.*, **30,** 185 (1927)
90. Keppie, J., Cocking, E. C., and Smith, H., *Lancet,* 246 (1958)
91. Pollitzer, R., *Plague,* 183 (Chronicle World Health Organization, Palais des Nations, Geneva, 698 pp., 1954)
92. Schar, M., and Meyer, K. F., *Schweiz. Z. allgem. Pathol. u. Bakteriol.*, 19, 51 (1956)
93. Schar, M., and Thal, E., *Proc. Soc. Exptl. Biol. Med.*, **88,** 39 (1955)
94. Girard, G., *Ann. Rev. Microbiol.*, **9,** 253 (1955)
95. Huddleson, F. I., *Michigan State University Centennial Symposium Report—Reproduction and Infertility,* p. 27 (Michigan State University Press, East Lansing, Mich., 1955)
96. Elberg, S. S., and Faunce, F., *J. Bacteriol.*, **73,** 211 (1957)
97. Wilson, G. S., and Miles, A. A., *Topley and Wilson's Principles of Bacteriology and Immunity,* 686 (Edward Arnold, Ltd., London, Engl., 2331 pp., 1955)

THE UNIVERSITY LIBRARIES OF GEORGIA

98. van Heyningen, W. E., *Bacterial Toxins,* 86 (Blackwell Scientific Publications, Oxford, Engl., 123 pp., 1950)
99. Wilson, G. S., and Miles, A. A., *Topley and Wilson's Principles of Bacteriology and Immunity,* 1720 (Edward Arnold, Ltd., London, Engl., 2331 pp., 1955)
100. Wilson, G. S., and Miles, A. A., *Topley and Wilson's Principles of Bacteriology and Immunity,* 1760 (Edward Arnold, Ltd., London, Engl., 2331 pp., 1955)
101. van Heyningen, W. E., *Bacterial Toxins,* 93 (Blackwell Scientific Publications, Oxford, Engl., 123 pp., 1950)
102. Ross, J. D., and Syverton, J. T., *Ann. Rev. Microbiol.,* **11,** 459 (1957)
103. Bell, S. D., and Johnson, R. E., *Proc. Soc. Exptl. Biol. Med.,* **92,** 46 (1956)
104. Jordan, W. S., Jr., *Proc. Soc. Exptl. Biol. Med.,* **92,** 867 (1956)
105. Berman, L., and Stulberg, C. S., *Proc. Soc. Exptl. Biol. Med.,* **93,** 730 (1956)
106. Westwood, J. C. N., Macpherson, I. A., and Titmuss, D. H. J., *Brit J. Exptl. Pathol.,* **38,** 138 (1957)

# PARASITIC DISEASES OF MAN (RECENT)[1]

By Ernest Carroll Faust[2]
*Tulane-Colombia Program in Medical Education,*[3]
*Facultad de Medicina, Universidad del Valle, Cali, Colombia*

## INTRODUCTION

For purposes of orientation it is important to distinguish between infection, e.g., the parasitism of one organism in or on another, and the actual or potential disease-producing capacity of the parasite with respect to its host (pathogenic index). The most prevalent ameba of man, *Entamoeba coli,* is apparently incapable of invading tissues; nor does its presence produce harm to the human body. In contrast, *Entamoeba histolytica* is potentially always a pathogen. In this review emphasis will be placed on protozoa and helminths which cause human disease.

## PROTOZOA PRODUCING HUMAN DISEASE

*Entamoeba histolytica.*—This ameba occurs in all populations of the world where surveys have been conducted (1 to 7) and its disease-producing effects are demonstrable in infections acquired in temperate climates (4, 8 to 11) as well as in the tropics. Yet, marked differences of opinion exist concerning the intrinsic pathogenicity of indigenous temperature zone strains. Many European workers contend that indigenous strains in their countries are nonpathogenic lumen inhabitants (12, 13, 14). One group attributes the presumed differences in pathogenicity to mean size measurements of two different races, a small nonpathogenic one with a mean cyst diameter of less than 10 μ and a large pathogenic race having a diameter in excess of 10 μ. Sapero *et al.* (15) first demonstrated that this difference was statistically significant. The writer (7) recently confirmed the existence of these two races in terms of size and morphologic characteristics. However, although the larger race is four times as prevalent as the small one in the tropical area in which this study was conducted, no remarkable evidence of pathogenicity was discovered in human infection with either race. In susceptible laboratory animals both races produce colitis when associated with suitable enteric bacteria (16 to 19). When *E. histolytica* was cultivated only with *Trypanosoma cruzi* and was inoculated into 35 bacterially-sterile guinea pigs, Phillips *et al.* (20) obtained no lumen infection or tissue invasion with this ameba, whereas 34 of 37 guinea pigs with normal enteric

[1] This review covers the relevant literature through November 30, 1957.

[2] William Vincent Professor of Tropical Diseases and Hygiene, Tulane University School of Medicine, New Orleans, La.; Visiting Professor, University of Valle, Cali, Colombia.

[3] International Cooperation Administration Mutual Assistance Program, U. S. Operations Mission to Colombia.

bacteria, inoculated in a similar manner, developed acute amebic ulcers of the cecum and the remaining three animals harbored *E. histolytica* as a lumen parasite. In the test tube and in the intestinal lumen of a susceptible animal, this ameba requires a living microörganism (21, 22, 23), but having gained entry into, and become lodged in living host tissues it can multiply and lyse tissues in a bacterially-sterile environment (24, 25, 26). The metabolic factor (enzyme) which the ameba apparently lacks but which is provided by certain enteric bacteria and by the cytoplasm of the host's body cells has not been identified.

Beaver *et al.* (27) carried out an extensive study on susceptibility to infection among 46 adult male human volunteers with a large race of *E. histolytica* obtained from an asymptomtac carrier. All challenged individuals, uninfected at the time of the experiment and fed viable cysts varying in number from 2000 to 1,000,000, became infected. The biological prepatent period was two to seven days in 68.7 per cent of the exposures and 9 to 14 days in all of the remainder except two, one of whom became positive on the 26th day and the other not until the 126th day. Seven of these volunteers apparently lost their infections spontaneously. Although there were no signs or symptoms attributable to the experimental human infections, inoculation of 10 dogs and 18 guinea pigs with this strain produced intestinal amebic ulceration in two and five instances, respectively. Undoubtedly, fewer than 2000 cysts of *E. histolytica* may at times produce natural human infection, and possibly even one cyst may lead to infection as it has in one case with *E. coli* (28).

Extensive studies have been conducted with human strains of *E. histolytica* in laboratory animals in an attempt (*a*) to correlate nutrition of the host with amebic infection (29 to 32); (*b*) to serve for screening of new drugs (33, 34, 35); and (*c*) to elucidate the conditions under which the parasite, after colonization in the intestinal wall, reaches and becomes established in the liver (36 to 39). Results have been contradictory with respect to different carbohydrate, protein, and vitamin requirements for *E. histolytica*. Rees (40) explains these apparent inconsistencies on the basis of varied enteric bacteria in experimental animals and the possibility that they are not suitable for such studies. Moreover, testing of drugs in experimental animals as *in vitro,* may not be applicable to human amebiasis. Chronic intestinal infection is a prerequisite for hepatic amebiasis (38). Moreover, Krupp (39), who fed infective-stage eggs of the doc ascarid *Toxocara canis* to guinea pigs and initiated visceral larva migrans before inoculating them with *E. histolytica,* found a significant increase in positive cultures of the organism from the liver tissue of these animals compared with controls which had not been exposed to *T. canis.*

In cultures of *E. histolytica* with undetermined bacterial associates, the life cycle of the organism has been elucidated and effects of the chemical agents, heat, and desiccation on the cysts have been determined (40). But metabolic activities of this ameba have been masked because of existing

symbiotic relationships. Monoxenic cultures with a known species of bacteria in defined media, survival in bacteria-free minced chick embryos, and growth with *T. cruzi* as associate have been achieved (41 to 44). Much information has been obtained on metabolic pathways of *E. histolytica,* employing cultured amebas with monoxenic and polyxenic bacterial associates, determining the activities of the complex and then subtracting those of the bacteria. This method has serious pitfalls but because "the attainment of axenic cultures seems so remote" it is presently the only practical procedure (45). Hallman & DeLamater (46) demonstrated amyolytic activity in *E. histolytica,* a result confirmed by Baernstein *et al.* (19), Kun & Bradin (47), Becker & Geiman (48), and others. Hilker *et al.* (49), employing chromatography, showed that amylase and maltase but not sucrase or lactase are present in lyophilized amebic preparations, thus presumably limiting the ability of this organism to utilize only carbohydrates which have a molecular structure of highly branched chains and providing a possible explanation for the inability of the ameba to utilize soluble starches in the medium. Kun *et al.* (50) conducted manometric analyses of washed suspensions and lysates of *E. histolytica* under anaerobic incubation. Following addition of various carbohydrates and sulfhydryl substrates, best yields of $CO_2$ and $H_2S$ were obtained from intact cells in the presence of glucose and cysteine with the addition of a co-factor extracted from rat liver. $CO_2$ was an end product of decarboxylization, there was mutual dependence on SH and hexose, while the degree of metabolic activity depended on the mass of amebas in the suspension. The bacteria did not contribute to production of $CO_2$ or $H_2S$ or the decarboxylization of pyruvate, but produced lactic acid (50).

Diagnosis of amebiasis remains a serious problem for physicians who do not have competently trained, experienced laboratory technicians. Fecal examination for detection of *E. histolytica* is the most dependable method (51, 52), but is complicated by fluctuating production and evacuation of the cysts (53), and by the fact that experts at times disagree concerning the differentiation of *E. histolytica* from *E. coli* in fecal films and from host tissue cells in proctoscopic aspirates (54). Brooke & Goldman (55) developed a polyvinyl alcohol technique for fixing fecal material in bulk or on slides, and Sapero & Lawless (56), combined a fixing and staining medium (merthiolate-iodine-formalin) for the satisfactory diagnosis of amebas and other protozoa. Goldman (57) distinguished between trophozoites of *E. coli* and *E. histolytica,* also between large and small races of *histolytica,* by use of fluorescein-tagged antiserum to large-race *histolytica.*

Practical immunologic techniques for diagnosis of *E. histolytica* have not been developed. In spite of earlier work by Craig (58) on complement fixation and refinements by Rees *et al.* (59, 60, 61), this test has not been standardized for routine clinical laboratory practice. It would be particularly useful in suspected cases of amebic hepatitis and as a follow-up of antiamebic therapy when fecal specimens are negative (62, 63).

Treatment of positive cases of *E. histolytica* infection constitutes an

important problem for physician and public health worker alike. The problem is complicated clinically by inadequate information concerning residual nonpatent infection following full courses of therapy (64), and from a public health point of view because of the menace of "asymptomatic carriers" in the propagation of amebiasis (65, 66, 67). Newer specific antiamebics for intestinal infection have recently been officially recognized, viz., bismuth glycoarsanilate and several antibiotics, including erythromycin and the tetracyclines; for hepatic and other types of extraintestinal amebiasis, chloroquine, which is as effective as emetine hydrochloride for this purpose and much safer (68 to 71). At times eradication of *E. histolytica* requires simultaneous treatment of enteric bacteria present in amebic lesions by employing antibiotics such as the tetracyclines (67).

In recent years chemotherapeutic prophylaxis has been undertaken for *E. histolytica* infections in "contained" communities. Craig (72) suggested that diiodoquin might be prophylactically effective in persons visiting highly endemic areas of amebiasis; Hauer (73) and Westphal (74) recommended bismuth glycoarsanilate to reduce infection in carriers. Berberian *et al.* (75), Hoekenga (76), and Sodeman & Beaver (70) demonstrated the preventive value of bismuth glycoarsanilate in subtherapeutic doses, and Beaver *et al.* (77) more recently provided similar proof of the efficacy of diiodoquin.

*Leishmanias and trypanosomes.*—All three human leishmanias are transmitted by the "bite" of sandflies (*Phlebotomus* spp.). These morphologically indistinguishable flagellates have been classified primarily on the type of disease which they produce in man, viz., *Leishmania tropica,* cutaneous infection; *Leishmania braziliensis,* mucocutaneous manifestations; and *Leishmania donovani,* visceral infection (kala-azar) (78). Earlier workers regarded these diseases as strictly of human origin (e.g., man-sandfly-man cycle) yet evidence indicates that they are, or have been, basically zoönotic (79). An ulcerative or moist variety of *L. tropica* infection is prevalent throughout extensive desert areas of Central South U.S.S.R., where the gerbil (*Rhombomus opimus*) is the vector (80), as it is in xerophytic regions of Iran (81). This type also exists in North Africa and French West Africa, where ground squirrels and gerbils are susceptible to experimental infection (79). The reservoir of "weeping" mucocutaneous leishmaniasis ("espundia") produced by *L. braziliensis* in rural Paraguay is possibly the agouti (*Dasyprocta aguti*) (82). In contrast, urban cutaneous leishmaniasis in Middle Asia has dry manifestations and apparently has become completely divorced from wild reservoirs. In Peru, natural infection with *L. braziliensis* occurs in dogs (83). Visceral leishmaniasis in China, Middle Asia, and the Mediterranean area is harbored by dogs and in Middle Asia also by jackals, both of which have cutaneous lesions and serve as constant sources of exposure for sandfly transmitters. In an endemic focus of kala-azar in northeastern Brazil dogs constitute urban reservoirs and the fox (*Lycalopex vetulus*) a suburban feral host (84).

The human trypanosomes of Africa, *Trypanosoma gambiense* and *Trypanosoma rhodesiense,* are transmitted by the "bite" of the tsetse fly (*Glossina* spp.) and the American species, *T. cruzi* and *T. rangeli,* by triatomid bugs (78). *Trypanosoma brucei* of wild game mammals in East Africa is regarded as the parent stock of the African species. *T. rhodesiense,* endemic in the high tablelands of East Africa and producing fulminating, typically fatal human disease, is considered to be transmitted from antelopes through wild (bush) tsetses to man. *T. gambiense* of West and Central Africa, urban in character and causing more chronic disease in man, has not been demonstrated in wild mammals, but domestic mammals are frequently infected and constitute a potential reservoir source (79). *T. cruzi,* distributed from northern Argentina and central Chile to southwestern United States, parasitizes many species of wild mammals throughout the entire area and many species of triatomid bugs are transmitters (85). The infective stage is deposited by the bug in liquid feces on the skin or mucous membrane of the mammal when the bug takes a blood meal. Human infection is geographically somewhat more restricted than that in mammalian reservoirs, is usually endemic in rural and suburban communities, and is transmitted by domestic triatomids. Dogs, cats, and cavies are common domestic reservoirs. Recently the first indigenous human infection was demonstrated in the United States (86). *T. rangeli* is found in man, dogs, and *Cebus fatuellus* in rural areas of Venezuela (87), Colombia (88), Guatemala (89), and possibly French Guiana (90). The vector is the triatomid *Rhodnius prolixus,* but unlike *T. cruzi, T. rangeli* is transmitted through the "bite" of this insect. Unlike other trypanosomes of man and vertebrate hosts, *T. rangeli* must migrate from the bug's midgut to the hemocele before it can accumulate in the salivary glands and proceed down the hypopharynx to be injected into the next mammalian host (91). *T. rangeli* multiplies in the mammalian host only in the trypanosome stage, but there is no evidence that it invades lymphatic or nervous tissue, and its course of infection is asymptomatic (91).

Hoare (79) considers that all of these blood and tissue flagellates of man are referable to three epidemiologic patterns, viz., (*a*) original natural infection in wild mammals and wild insect transmitters; (*b*) a situation in which man in rural areas near an enzoötic focus acquires the infection; and (*c*) urbanized epidemiologic infection in which man, domestic reservoirs, and vectors are involved. In many respects this parallels the epidemiology of yellow fever (92) and plague (93).

Chang (94) studied electron microscope pictures of the leishmania and leptomonas stages of *L. donovani.* In the former, the nucleus was enclosed by a smooth outer and a ridged inner membrane, while the leptomonas-stage nucleus was provided only with the smooth membrane. The fibrillar axoneme, surrounded by a helically-coiled band of cytoplasm, originates proximally from a rounded basal granule (kinetoplast); in the leptomonas stage the coiled band extends to the distal end of the flagellum. Previously

Das Gupta *et al.* (95) reported a maximum of nine fibrils in the flagellum of *L. donovani.* Similar flagellar structure has been described for *L. tropica* (96), *T. lewisi,* and *T. brucei* (97, 98) and *T. cruzi* (99), although the number of fibrils varies in different genera and species, and at times within the same species.

Culture forms of *L. tropica* and *T. cruzi* manifest increased respiration compared with the endogenous rate when Krebs cycle intermediates are added to the medium; malonate inhibits endogenous respiration but this effect is countered by the addition of succinate; *L. tropica* is more sensitive than *T. cruzi* to ionic and pH changes and to impurities in carboxylic intermediates (100). Working with *T. cruzi,* Yaeger (101) reported that "metabolically the culture and intracellular stages of this organism more nearly resemble one another than they do the bloodsteam form, with two notable exceptions," viz., the cultured trypanosomes do not utilize any appreciable amount of lactate, nor do pyruvate and lactate accumulate appreciably in the presence of glucose. The bloodstream organisms consume more $O_2$, glucose, pyruvate and lactate, and produce more lactate and pyruvate from glucose. All three stages are equally sensitive to cyanide and arsenite. *T. rhodesiense,* when maintained in laboratory animals by subinoculation for only six months, greatly increases its oxidative metabolism, loses its ability to infect man, guinea pigs, and tsetse flies, and loses its polymorphism (102). Its prepatent period is greatly reduced compared with the natural cycle involving alternate passage through the tsetse (102). This may explain conflicting results obtained in tests on drug sensitivity carried out under such unnatural laboratory conditions. In a study of the mechanism of immunity developed in *T. cruzi* infection in the experimental host, Pizzi (103) concluded that the primary role of resistance to the parasite resides in the phagocytic capacity of the connective tissue macrophages, and that antibodies do not participate directly in this host-parasite reaction.

*Toxoplasma gondii.*—This intracellular one-celled organism, which divides by logitudinal binary fission, is commonly regarded as a protozoön (104, 105). Jacobs (104) described it as "crescentic or arc-shaped," with one end more attenuated than the other, measuring 4 to 7 μ by 2 to 4 μ, with a vesicular nucleus, and lacking a neuromotor apparatus. Gustafson *et al.,* (106), studying electron microscope sections (0.05 μ or less), described a hollow "conoid" organelle within the cytoplasm, having its distal end associated with the more pointed end of the cell membrane and its open base near the center of the cell; and associated with the "conoid" 14 to 18 solid fibrils ("toxonemes"), attenuated distally and coarser proximally, extending into the cytoplasm of the cells. *Toxoplasma* utilizes the Krebs cycle of oxidative metabolism, with glucose as a substrate and evolution of $CO_2$. Respiration is 85 per cent inhibited by cyanide (107).

As an obligate intracellular parasite, *T. gondii* has a wide range of mammalian hosts from marsupials to primates, several species of birds, and, reportedly, reptiles and a frog (104). Experimental animals may become

infected by oral, intranasal, intravenous, or intraperitoneal inoculation. Following multiplication at the site where the inoculum becomes first established, there is general dissemination through the blood stream, with later more chronic infection in host cells as antibodies build up and the parasitemia disappears (108).

Although the earliest described human cases of toxoplasmosis were severe, often fatal, and acquired prenatally, probably not more than one in 35,000 births has this stigma (109). During the acute stage of human toxoplasmosis, adenopathy, with or without fever, may be the only clinical sign (110).

Toxoplasmosis is global in its distribution but is more prevalent in warm moist climates than in those which are cold or dry (104). Estimates of the prevalence of toxoplasmosis in man are commonly based on immunologic tests, principally the Sabin-Feldman dye test (111) or on complement-fixation (112). Cathie (113) weighed evidence concerning cross reactions obtained by these tests with *Trichomonas vaginalis*, human trypanosomes, plasmodia, *Salmonella typhi*, and sarcosporidia. He concluded that "much of the experimental animal work on toxoplasmosis is not applicable to man," and that the dye and complement-fixation reactions are valid for human sera. Jacobs & Lunde (114) regard the dye test as presently "the most useful diagnostic procedure for toxoplasmosis." However, it is not ideal for clinical laboratory diagnosis because it is difficult to perform, requires live parasites, has the risk of human laboratory infection, and necessitates careful manipulation. These workers (114) adapted a hemagglutination test for toxoplasmosis and obtained good correlation with the dye test, especially when pooled antigens were employed.

Human infections have been reported in association with infected domestic animals (115, 116, 117). In England, Beverley *et al.* (118), employing the dye test, found 2 per cent positives in normal adults, 12 per cent among veterinarians and slaughter-house workers, 43 per cent in rabbit dressers, and 67 per cent in rabbit trappers. In England and Germany dye-test positives among rural inhabitants are significantly more common than in the urban population of the same districts (109, 119), but in Oslo the reverse is true (120). Although Feldman (121) found three-fourths of Tahitians positive by nine years of age, there was no evidence of congenital transmission. Experimental animals have become infected from a variety of inocula, including infected raw meat, and by a variety of routes (104, 109). Although the methods of transmission to man are not understood, close association with infected animals or persons in an active stage of the disease is probably a determining factor (116, 117, 118, 122, 123). Arthropod transmission may be possible (124, 125), but does not seem to be a common source of exposure.

During the acute stage, the toxoplasmas proliferate in parenchyma cells as well as fixed macrophages, leading to focal necrosis, the continuing amount of which depends on the virulence of the strain, the host's ability

to develop immune bodies, and the capacity of the macrophages to destroy the parasites rather than to serve as host cells (126). This latter reaction accounts for granulomas in the brain, retina, and other sites, and the relatively rare terminal calcification in the central nervous system and chorioretinal tissues (127). So-called pseudocysts develop during the height of the acute proliferative stage when immunizing bodies are becoming operative (126). These masses of toxoplasmas within greatly enlarged host cells are found in various organs and tissues of the host's body, including the retina and brain. Although the toxoplasmic lesions are frequently miliary, they may be so widely dispersed in different organs and tissues that a long search is required for their discovery. A majority of patients with an acquired mild infection exhibit regional or more general lymphadenopathy, with or without fever (109), occasionally with a petechial rash (109). In a majority of these clinical cases, a specific diagnosis depends on the dye and complement-fixation tests. Experimental tests in laboratory animals and in a few laboratory-acquired human infections indicate that sulfa drugs, antibiotics, and pyrimethamine may be useful in the early acute stage (129).

*Pneumocystis carinii.*—This organism, probably a protozoön but of uncertain relationship, has been recovered from the mouse, rat, rabbit, guinea pig, dog, fox, goat, sheep, and man. Human cases were earlier described only from Central Europe (130, 131), principally in the newborn. More recent reports have come from the United States (132, 133), Canada and Chile (134). The organism can not be easily identified in tissue sections but in impression smears from infected lungs it is usually found free in frothy alveolar exudates, rarely in macrophages and interstitial cells (134, 135, 136). Pizzi & Díaz (134) described three arrangements of the organism, (*a*) isolated uninucleate forms; (*b*) isolated forms with more than one nucleus; and (*c*) rosettes composed of eight organisms within a common envelope. Each measures 1 to 3 μ in length, is rounded, oval, slightly irregular, or rarely elongated crescent-shaped. These parasites lack cytoplasmic granules and both extremities are equally pointed (134), a fact which distinguishes *Pneumocystis* from *Toxoplasma.* The most characteristic pattern of *Pneumocystis* is the rosette, 7 μ in diameter, rounded and enveloped by a delicate membrane (134, 136).

*Pneumocystic* infection produces the clinical entity "interstitial plasma-cell pneumonia" typically found in premature or immature debilitated infants. This consists of extensive plasma-cell infiltration in and around pulmonary alveoli, with a frothy, honeycombed exudate (137, 138). The disease is contagious and may develop epidemically in children's wards in a hospital. It has an insidious or sudden onset, with profound dyspnea, pallor and anguished facies, lacking distinct auscultatory evidence of pneumonitis (138). Its usual duration is one to two months, with sudden, unexpected death, yet isolated recoveries have been reported (139). No satisfactory chemotherapy has been developed. Pizzi & Díaz (134) suggest that infection is acquired by debilitated individuals who lack properdin, the serum protein

believed to play a role in immunity (140). Complement-fixation tests for *Pneumocystis* infection have been confirmed by recovery of the parasite at autopsy (141).

*Malaria parasites.*—The life history of the malaria parasites of man, including the pre-erythrocytic development of these plasmodia in the parenchyma of the liver, has been presented by Garnham in Volume 8 of this *Annual Review* (142). In studying the patho-physiologic processes of malaria, Maegraith *et al.* (143) found that (*a*) anoxemia, caused by the destruction of infected and unparasitized red blood cells, and (*b*) anoxia, with stagnation of blood in cerebral capillaries and increased permeability of their walls, together with construction of blood vessels in the liver, constitute the dynamics of the clinical picture of malaria, especially that produced by *Plasmodium falciparum.*

Recent studies in Africa on the relationship of the sickling-cell trait to *falciparum* malaria indicate that in nonimmune individuals the trait carriers are as susceptible as those with normal hemoglobins. However, sickle-cell hemoglobin (S) which has valine instead of glutamic acid as its amino acid component and forms rigid polymers when deoxygenated, appears to limit multiplication of the plasmodia, thus reducing the mortality rate in these subjects compared with infected persons having normal hemoglobin. This favors survival of the infected persons with hemoglobin S and of the sickle-cell trait, which is heterozygous and behaves as a single Mendelian character (144).

An ideal antimalarial drug should (*a*) destroy all asexual forms of the parasite in the blood stream, curing the clinical attack; (*b*) eradicate all exoerythrocytic forms, thus preventing relapse; and (*c*) kill all gametocytes to forestall infection of the *Anopheles* mosquito [(145) p. 283]. No single drug has all these attributes but a combination of chloroquine and primaquine serves this threefold purpose. For suppressive and clinical cure, chloroquine is probably the more effective and well-tolerated antimalarial. Moreover, it eradicates *falciparum* parasites which presumably have no residual exoerythrocytic focus (146). Chlorguanide (147), a pyrimidine derivative, although well-tolerated in curative doses, a good suppressant in all forms of malaria and a prophylactic in *falciparum* malaria, acts more slowly than chloroquine in relieving symptoms, and produces a drug resistance of parasites (146), including gametocytes (148). Pyrimethamine, another pyrimidine derivative, has excellent suppressive action in very small amounts, eradicates *P. falciparum* but produces parasite drug resistance, including a cross-resistance to chlorguanide (149). The 8-aminoquinolines are the most effective drugs in eradicating exoerythrocytic foci of *Plasmodium vivax* and *Plasmodium malariae*, as well as the sterilization of gametocytes. The most recently developed member of this group is primaquine, which is the least toxic in effective doses (150).

Control of malaria was almost a superhuman task until the development of the more effective antimalarial drugs and the employment of DDT

as an antilarval and anti-imagicidal weapon against *Anopheles* mosquitoes. Although, in 1955, some 375 million persons were under protective measures, about 700 million were still subject to exposure and 200 million clinical cases and 2 million malaria deaths were reported (151). In 1955 the Eighth World Health Assembly recommended world-wide malaria eradication as ultimately cheaper and more effective than control. Many malarious countries are still in the preparatory phase of this program, others have started the attack phase, and some, including the United States and several tropical countries, have already achieved the desired goal (151). Because of developing resistance to DDT or avoidance reaction by *Anopheles* mosquitoes (152, 153, 154), time for a concentrated attack is running out and, if allowed to lapse, the opportunity for world-wide eradication of malaria may never again be afforded.

## HELMINTHS PRODUCING HUMAN DISEASE

### Nematodes (true roundworms)

*Larva migrans.*—The term connotes a functional rather than a systematic grouping of immature metazoa, especially nematodes, which invade human tissues but for which man is typically an unnatural host. Following exposure and invasion of the human body, these larval nematodes may undertake migration through the tissues but commonly fail to mature or complete their journey, and in some infections are destroyed by host-cell infiltration. For convenience, larva migrans caused by nematodes may be classified as: (*a*) cutaneous, produced by hookworms and *Strongyloides;* (*b*) visceral, produced by *Toxocara canis*; (*c*) filarial larva migrans; and (*d*) spiruroid larva migrans (155).

*Cutaneous larva migrans.*—This disease most frequently results from invasion of the human skin by infective-stage larvas of the tropical and semitropical dog hookworm, *Ancylostoma braziliense.* The etiology of this disease was first elucidated in Jacksonville, Florida (156), in the intensively enzoötic-endemic area which extends throughout the coastal regions of Florida and along the Mississippi Gulf Coast. Here, thousands of persons contract the disease annually during summer vacations (157, 158). Hookworms less frequently involved, and possibly less capable of producing the characteristic picture of cutaneous larva migrans in most exposed persons, are the temperate zone *A. caninum* (159, 160, 161) and the European *Uncinaria stenocephala* (162) of dogs; the cattle hookworm, *Bunostomum phlebotomum* (163); and the human *A. duodenale* and *Necator americanus* (159, 164). Naturally acquired larva migrans caused by *A. braziliense* results from contact with moist sandy soil where infected dogs and cats have previously defecated and the hatched larvas have developed to the infective stage (157, 158). Infection from the other species consists of isolated cases of natural, accidental, or experimental cutaneous exposure (164). The reservoir-host strains of *A. braziliense* typically never reach cutaneous blood vessels of man to pursue a normal course of migration but migrate

in raised serpiginous tunnels in the skin. In accidental and experimental infections with the other hookworms mentioned above, the larvas may fail to penetrate the skin (159), the infection may be abortive in a few days (163, 164), or there may be a typical sequel of cutaneous larva migrans (160, 164).

Cutaneous creeping eruption produced by *Strongyloides stercoralis* is autoinfective in origin and perianal in its location. Rhabditoid larvas of *S. stercoralis,* hatched from eggs in the wall of the human intestine, are evacuated in the feces usually as second-stage rhabditoid larvas (165). But, at times, they transform to the infective stage in the intestinal lumen, causing internal autoinfection (166) or, lodged in moist feces on the anus, produce perianal autoinfection (167). This latter situation may result in radiating migration of the larvas in tunnels within the skin, while similar lesions on the abdomen, chest, and shoulders may have resulted from this type of autoinfection (168, 169). These unusual cases of *Strongloides* autoinfection have been reported only for European prisoners of war in Thailand and Malaya, under conditions of primitive personal hygiene and malnutrition (168, 169).

*Visceral larva migrans.*—This syndrome is a concept of recent origin. Although clinical cases which fit this syndrome were reported in 1947 (170); 1949 (171); 1950 (172, 173); and 1953 (174) from the United States and in 1951 (175) from England, the disease entity was first distinguished in 1952 by Beaver *et al.* (176). Cases have since been diagnosed from New England to California and from Minnesota to Louisiana (164). The syndrome as originally defined (176) consists of persistent chronic eosinophilia of marked degree and granulomatous lesons in the livers of young children, accompanied by appreciable pulmonary infiltration, and caused by dog and cat ascarids of the genus *Toxocara.* While the original clinical picture is accurate with respect to the usual age group involved, the concept requires extension to include the eye (173) and other viscera (175), affecting various age groups, while the only species of *Toxocara* which has been identified in human visceral larva migrans is *T. canis.* Infection results from ingestion of infective-stage eggs of this nematode obtained from soil contaminated with feces of infected dogs. The eggs hatch in the human duodenum and the emerging larvas begin migration to the lungs as in their natural host. En route they are commonly trapped in the liver, but may reach the lungs, brain, eyeball or other soft tissues, where they are surrounded by infiltration of host cells, producing a pseudotubercle around each larva. In experimental and human material, Nichols (177) obtained no evidence of growth or metamorphosis characteristic of the migration period in normal hosts. The comparative morphologic studies of this worker on larvas of *Toxocara* (177), and of *Ascaris lumbricoides, Necator americanus, Ancylostoma caninum* and *Strongyloides stercoralis* (178) furnish a dependable guide for identifying these nematodes in human tissues.

No standard immunologic diagnostic test has been perfected for visceral

larva migrans (164). Fatalities have occurred at times in small children with miliary infections, especially in the lungs and brain, but usually the infection runs a course of weeks or months, with eventual recovery. No specific treatment has been discovered.

*Filaria larva migrans.*—This is the third type of immature nematode migrating in the tissues of man as abnormal host. A majority of the worms from the earlier cases reported were immature females in subcutaneous nodules in various parts of the body (155); while recognizable as filarias they were not specifically diagnosable. Adults closely related to, if not actually the same as canine dirofilarias have, on several occasions, been described from the human host (179 to 185). Desportes (186) placed the immature filarias in the genus *Dirofilaria* and, because the name *conjunctivae* had first been employed in connection with these forms (187), he referred to them as *D. conjunctivae.* Except for one case from Argentina (188), the earlier discoveries came from the Mediterranean area and eastward to India (189). In 1952, Faust *et al.* (190) reported two cases from Florida. More recently, Faust (189) added one and Beaver (191) has a record of two additional cases from Florida. Meanwhile, it has been demonstrated (190) that the first three specimens from Florida and one from Thailand (189) are referable to *Dirofilaria* (subgenus *Nochtiella*). Present information is insufficient to determine whether or not the immature specimens of *D. conjunctivae* from man are identical with the subcutaneous dog filuaria *D. repens,* or represent different species.

*Spiruroid larva migrans.*—This is the fourth type of migration of immature nematode through human tissues. Members of the superfamily (Spiruroidea) are distantly related to the filarias (Filarioidea). Within the spiruroids several instances of human infection with mature and immature specimens of migrating *Gongylonema* (192, 193), and one case of subcutaneous abscess formation caused by a species of *Physaloptera* (194) have been reported. More commonly, immature forms of *Gnathostoma spinigerum* are recovered from tunnels and abscesses in superficial and deep tissues, including records from India (195); Thailand (196, 197, 198); Indonesia (199); Indochina (200); China (201); the Philippines (202, 203); Japan (204, 205); and one from Palestine (206). Man acquires infection from eating raw infected freshwater fish, the second intermediate host. Cats and their wild relatives are the normal definite hosts, in which the worms develop to adults in stomach tumors (196, 197).

*Reservoirs of human filarias.*—In contrast to filaria worms which are normally parasitic in other animals and are occasionally found in man, several species of filarias are specifically adapted to the human host. Thus far there is no evidence that *Wuchereria bancrofti* and *Onchocerca volvulus* have reservoir hosts, but cumulative data suggest that *Wuchereria malayi, Loa loa* and *Acanthocheilonema streptocerca* may have reservoirs in wild mammals. In Malaya, wild *Macaca cynomolga* is infected with microfilarias resembling *W. malayi* of man, and *Mansonia* mosquitoes serve equally well

as intermediate hosts of the simian and human strains (207, 208). Experimentally this filaria has been transmitted to cats (208). In rain forests of tropical Africa a filaria indistinguishable from *Loa loa* is found only in simian hosts; in other localities where monkeys are not present the infection is probably confined to man as definitive host, and in certain other localities the biological vectors, *Chrysops* spp., may transfer the filaria from monkeys to man and vice versa (209, 210, 211). *A. streptocerca,* a frequent parasite of man in tropical Africa, has been described in its adult stage only from chimpanzees (*Pan paniscus, Pan satyrus*), with the gnat *Culicoides grahami* as intermediate host and vector (212, 213).

*Axenic* in vitro *development of nematode parasites.*—Earlier studies of larval and adult nematodes under axenic conditions were concerned primarily with survival of the organisms (214). Later, larval development and beginning differentiation into adults were obtained for strongyloid species (215 to 218) and for *Trichinella spiralis* (219), while slight growth was demonstrated in larvas artificially hatched from *A. lumbricoides* eggs (220). The first almost complete *in vitro* development of a mammalian nematode under bacteriologically sterile conditions was accomplished by Weinstein & Jones (214). Employing *Nippostrongylus muris* of the rat in axenic cell-free media in roller-tube cultures, these investigators obtained growth through the third larval stage in 50 per cent chick embryo extract; further metamorphosis to sexually mature adults required a medium containing caseinate or casein hydrolysate, filtrate of powdered yeast or liver extract, chick embryo extract and mammalian serum. Mating of adults was not observed and development in culture required four- to fivefold the time for that in the natural host.

## Trematodes (Flukes)

*Blood flukes* (*schistosomes*).—In 1952, the writer (221) referred to human blood fluke infection as "one of the devastating diseases of mankind, second only to malaria in its deleterious effects on the social and economic development of populations in many warm climates of the world." Four etiologic agents, *Schistosoma japonicum* in the Far East, *Schistosoma mansoni* in Africa and the American Tropics, *Schistosoma haematobium* throughout Africa and extending to western India, and *Schistosoma intercalatum* in a limited area of tropical Africa, are responsible for human schistosomiasis, which is contracted from fresh water containing the cercarias which escape from the snail intermediate hosts. Infection is increasing geographically owing to the introduction of infected snails into new areas and by immigration of infected persons into previously uninfected territory in which susceptible snails breed (222 to 225). *S. japonicum* produces natural infection in most mammals which frequent cercaria-infested water. While several species of rodents and other mammals are occasional natural hosts of *S. mansoni* (226 to 229), except for *S. japonicum* man is the only important reservoir of these blood flukes (230, 231).

Kuntz (232) grouped blood flukes of man and other natural mammalian hosts in "complexes," viz., *S. haematobium* and *S. intercalatum* of man with *Schistosoma bovis* of cattle and possibly *Schistosoma matthei* of sheep, *Schistosoma curassoni* and *Schistosoma spindalis* of cattle; *S. mansoni* of man with *S. mansoni* var. *rodentorum* and *Schistosoma rodhaini* of African rodents; and endemic strains of *S. japonicum* with the Formosan enzoötic strain of this species, which is incapable of maturing in the human host (233) and of infecting Formosan macaques, but is able to infect Philippine and Japanese macaques (234). Geographic isolation, even between Egypt and West Africa (*S. haematobium*), was believed responsible for refractoriness of the Egyptian snail, *Bulinus truncatus,* to the West African strain of the parasite (235); similarly a strain of *Australorbis glabratus* from São Salvador, Bahia, Brazil, proved completely refractory to infection with a Puerto Rican strain of *S. mansoni* which produces 95 per cent infection in Puerto Rican *A. glabratus* (236). Newton (237) crossed these two strains of *A. glabratus* and demonstrated that susceptibility to infection is a heritable character involving several genetic factors. Barbosa *et al.* (238) also found that geographically isolated molluscan hosts of *S. mansoni* interbreed, producing fertile progeny, and concluded that these "snails from Egypt and Brazil have a common gene pool."

Several workers have studied hermaphroditic tendencies in human and related schistosomes: in *Schistosoma* the female organs are invariably in the males (239, 240, 241), whereas in *Schistosomatium* (*S. douthitti*) the testes are in the females (242). In none of the specimens studied were the reciprocal organs functional (242).

All human blood flukes stimulate antibody production (243). The mammalian host produces specific antibodies to three stages of the parasite, viz., cercaria, adult, and egg (244). Complement-fixation and precipitin tests, with antigen from cercarias and adult worms, have been employed for many years (243, 244). Serologic tests with immune sera and living cercarias have caused precipitin reaction with fresh serum (245), and a hood-like envelope if the serum is heated (246). Cercaria, adult and circumoval precipitins, and cercarial agglutinins have been obtained with serum from *S. mansoni* patients (244). The cercarial tests are most pronounced in earlier infections; circumoval reactions are more common in sera from chronic cases. Stirewalt & Evans (247), testing the effect of sera from uninfected and infected laboratory mammals on living cercarias of *S. mansoni,* concluded that cercaricidal activity and precipitate formation are not related to schistosome infection in these hosts, and that the hood-like pericercarial envelope develops in cercaricidal sera only after heat inactivation. Kagan (248) reported that miracidial immobilization is more sensitive than the pericercarial envelope reaction in animals infected or immunized with schistosome antigens.

Control of schistosome infections "is a complicated problem, much more

difficult to solve than the control of malaria, urban yellow fever, epidemic typhus and the leishmaniases" (221). The two major objectives are (*a*) development of public health consciousness among populations in endemic areas (ultimate goal), and (*b*) immediate measures to provide safe water for domestic use, naturalistic methods of snail control, and application of molluscicidal chemicals. A large number of synthetic compounds have been tested to determine their practical usefulness as molluscicides (249). Sodium pentachlorophenate and dinitro-6-cyclohexylphenol have proved to be the most effective in extensive laboratory and field trials in Japan (250), the Philippines (251, 252), Egypt (253), Brazil (254), and the Dominican Republic (255). The ecologic problem of snail control varies with topography of the country, type of water in which the snails breed, and habits of the respective snail hosts in different areas (256). Olivier (254) concluded that "in most endemic areas, eradication is probably not attainable in the near future," and that "lasting control of schistosomiasis in many endemic areas where aquatic vectors are involved will be effected only after improvement in environmental sanitation and improvement of water supplies."

*Schistosome cercarial dermatitis.*—This syndrome, demonstrated 30 years ago to result from contact with cercarias of avian blood flukes in a freshwater lake in Michigan (257), is essentially world-wide in distribution, not only in fresh water (258 to 261) but also along marine shores (262, 263, 264). In addition, mammalian schistosome cercarias not infective for man have been incriminated (265, 266). The known species of bird schistosomes belong to the genera *Trichobilharzia, Gigantobilharzia, Ornithobilharzia,* and *Microbilharzia* (vel *Austrobilharzia*) (258, 259, 261, 262, 263), and the known mammalian species, to *Schistosoma* (265, 266) and potentially *Schistosomatium* (267). Olivier (268) and Macfarlane (260) obtained evidence that cercarial dermatitis is a sensitization reaction, while Hsü & Ameel (269) demonstrated that antigens prepared from human schistosomes are not diagnostically helpful in differentiating cercarial from other types of sea-bather's dermatitis.

## Cestodes (Tapeworms)

*Echinococcus miltilocularis.*—For many decades *E. granulosus* was the only recognized species of the tapeworm producing hydatid cysts in man and domestic mammals. Yet distinct morphologic types are found in human cases, viz., the widely disseminated unilocular type, the osseous type, and the alveolar type which occurs primarily in Central Europe and eastward to Siberia and Japan. Cameron (270) explained the alveolar type as the result of breaking out of germinal tissue from the outer membrane and capsular sheath of the hydatid. Rausch & Schiller (271), Rausch (272), and Thomas *et al.* (273), all working in Alaska, and Vogel (274, 275) in Southern Germany, demonstrated the complete life cycle of the alveolar type. In the Arctic regions the larval stage occurs in the tundra vole (*Microtus*

*oeconomus*), field mouse (*Clethrionomys rutilis*), ground squirrel (*Citellus undulatus*) and shrew (*Sorex jacksoni*), and the adult *Echinococcus* in the Arctic fox (*Alopex lagopus*), wolves, and dogs. In Germany the field mouse (*Microtus arvalis*) and red fox (*Vulpes vulpes*) are the respective larval and adult hosts. Man is exposed sporadically by ingesting eggs evacuated in the feces of foxes and other definitive hosts (274). Experimental infection in rodents indicates that the larva of the alveolar type is invariably uncircumscribed and produces a metastasizing malignancy with fatal termination, as is the case in man (276). Morphologic differences in the adult stage between *E. granulosus* and that of the alveolar type, as well as morphologic and physiopathologic differences in the hydatid stage, indicate that the alveolar type is a separate species which should be designated as *E. multilocularis* [(Leuckart, 1863), (syn. *E. sibiricensis* Rausch & Schiller, 1954), (277)].

## LITERATURE CITED


1. Miller, M. J., and Choquette, L. P. E., *Can. J. Research,* **E, 25,** 1–4 (1947)
2. Hitchcook, D. J., *J. Parasitol.,* **36,** 232–34 (1950)
3. Castex, M. R., and Greenway, D., *Prensa méd. (Argentina),* **21,** 2049–70 (1934)
4. Ritchie, L. S., and Davis, C., *Am. J. Trop. Med.,* **28,** 803–16 (1948)
5. Elsdon-Dew, R., *Am. J. Trop. Med.,* **29,** 337–40 (1949)
6. Kessel, J. F., Parrish, M., and Parrish, G., *Am. J. Trop. Med. Hyg.,* **3,** 440–46 (1954)
7. Faust, E. C., *Am. J. Trop. Med. Hyg.,* **7,** 4, 15 (1958)
8. Bundesen, H., *Public Health Repts. (U.S.),* **49,** 1266–72 (1934)
9. Hardy, A. V., *Public Health Repts. (U.S.),* **50,** 323–34 (1935)
10. Morton, T. C., Stamm, W. P., and Seidelin, R., *Brit. Med. J.,* **II,** 114–16 (1952)
11. Brooke, M. M., Melvin, D. M., Sappenfield, R. W., Payne, F. M., Carter, F. R. N., Offutt, A. C., and Frye, W. W., *Am. J. Hyg.,* **62,** 214–26 (1955)
12. Brumpt, E., *Précis de Parasitologie,* 217–26 (Masson & Cie, Paris, France, 1042 pp., 1949)
13. Nauck, E. G., *Lehrbuch der Tropenkrankheiten,* 113–28 (Georg Thieme Verlag, Stuttgart, Germany, 432 pp., 1956)
14. Hoare, C. A., *Exptl. Parasitol.,* **1,** 411–27 (1952)
15. Sapero, J. J., Hakansson, E. G., and Louttit, C. M., *Am. J. Trop. Med.,* **22,** 191-208 (1942)
16. Faust, E. C., Lewis, R., Heilbrunn, I., Hickman, M. L. W., and Everitt, M. G., *Rev. méd. (México),* 206–10 (1948)
17. Tobie, J. E., *Proc. Soc. Exptl. Biol. Med.,* **45,** 691–93 (1940)
18. Carrera, G. M., and Faust, E. C., *Am. J. Trop. Med.,* **29,** 647–67 (1949)
19. Baernstein, H. D., Rees, C. W., and Reardon, L. V., *Am J. Trop. Med. Hyg.,* **3,** 839–48 (1954)
20. Phillips, B. P., Wolfe, P. A., Rees, C. W., Gordon, H. A., Wright, W. H., and Reyniers, J. A., *Am. J. Trop. Med. Hyg.,* **4,** 675–92 (1955)

21. Chinn, B. D., Jacobs, L., Reardon, L. V., and Rees, C. W., *Am. J. Trop. Med.*, **22,** 137–46 (1942)
22. Phillips, B. P., *Science,* **111,** 8–9 (1950)
23. Cleveland, L. R., and Sanders, E. P., *Am. J. Hyg.*, **12,** 569–605 (1930)
24. Cleveland, L. R., and Sanders, E. P., *Science,* **72,** 149–51 (1930)
25. Carrera, G. M., *Arch. Pathol.*, **50,** 440–49 (1950)
26. Reinertson, J. W., and Thompson, P. E., *Proc. Soc. Exptl. Biol. Med.*, **76,** 518–21 (1951)
27. Beaver, P. C., Jung, R. C., Sherman, H. J., Read, T. R., and Robinson, T. A., *Am. J. Trop. Med. Hyg.*, **5,** 1000–9 (1956)
28. Rendtorff, R. C., *Am. J. Hyg.*, **59,** 196–208 (1954)
29. Taylor, D. J., and Greenberg, J., *Federation Proc.*, **10,** 396 (1951)
30. Sadun, E. H., Bradin, J. L., Jr., and Faust, E. C., *Am. J. Trop. Med.*, **31,** 426–37 (1951)
31. Taylor, D. J., Greenberg, J., and Josephson, E. S., *Am. J. Trop. Med. Hyg.*, **1,** 559–66 (1952)
32. Carrera, G. M., Sadun, E. H., and Faust, E. C., *Am. J. Trop. Med. Hyg.*, **1,** 966–69 (1952)
33. Jones, W. R., *Proc. Intern. Congr. Trop. Med. & Malaria, 4th Congr.*, **2,** 1088–99 (1948)
34. Hrenoff, A. K., *J. Parasitol.*, **37,** 329–30 (1951)
35. Thompson, P. E., and Reinertson, J. W., *Am. J. Trop. Med.*, **31,** 707–17 (1951)
36. Reinertson, J. W., and Thompson, P. E., *Proc. Soc. Exptl. Biol. Med.*, **76,** 518–21 (1951)
37. Rees, C. W., Taylor, D. J., and Reardon, L. V., *J. Parasitol.*, **40,** 390–91 (1954)
38. Maegraith, B., and Harinasuta, C., *Ann. Trop. Med. Parasitol.*, **48,** 421–41 (1954)
39. Krupp, I. M., *Exptl. Parasitol.*, **5,** 421–26 (1956)
40. Rees, C. W., *Problems in Amebiasis* (Charles C Thomas, Publisher, Springfield, Ill., 119 pp., 1955)
41. Rees, C. W., Baernstein, H. D., Reardon, L. V., and Phillips, L., *Am. J. Trop. Med. Hyg.*, **2,** 1002–14 (1953)
42. Shaffer, J. G., Ryden, F. W., and Frye, W. W., *Am. J. Hyg.*, **49,** 127–33 (1949)
43. Shaffer, J. G., Sienkiewicz, H. S., and Washington, J. E., *Am. J. Hyg.*, **57,** 366–79 (1953)
44. Phillips, B. P., *Science,* **111,** 8–9 (1950)
45. Ballamuth, W., and Thompson, P. E., 277–345 in *Biochemistry and Physiology of Protozoa,* II (Academic Press, Inc., New York, N.Y., 388 pp., 1955)
46. Hallman, F. A., and DeLamater, J., *Exptl. Parasitol.*, **2,** 170–73 (1953)
47. Kun, E., and Bradin, J. L., Jr., *Biochim. et Biophys. Acta,* **11,** 312–13 (1953)
48. Becker, C. E., and Geiman, Q. M., *Exptl. Parasitol.*, **4,** 493–501 (1955)
49. Hilker, D. M., Sherman, H. J., and White, A. G. C., *Exptl. Parasitol.*, **6,** 459–64 (1957)
50. Kun, E., Bradin, J. L., Jr., and Dechary, J. M., *Biochim. et Biophys. Acta,* **19,** 153–59 (1956)

51. Faust, E. C., Sawitz, W., Tobie, J. E., Odom, V., Peres, C., and Lincicome, D. R., *J. Parasitol.*, **25**, 244–62 (1939)
52. Tobie, J. E., Reardon, L. V., Bozicevich, J., Shih, B. C., Mantel, N., and Thomas, E. H., *Am. J. Trop. Med.*, **31**, 552–60 (1951)
53. Lincicome, D. R., *Am. J. Hyg.*, **36**, 321–37 (1942)
54. Brooke, M. M., Otto, G. F., Brady, F., Faust, E. C., Mackie, T. T., and Most, H., *Am. J. Trop. Med. Hyg.*, **2**, 593–612 (1953)
55. Brooke, M. M., and Goldman, M., *J. Lab. Clin. Med.*, **34**, 1554–60 (1949)
56. Sapero, J. J., and Lawless, D. K., *Am. J. Trop. Med. Hyg.*, **2**, 613–19 (1953)
57. Goldman, M., *Am. J. Hyg.*, **59**, 318–25 (1954)
58. Craig, C. F., *Am. J. Trop. Med.*, **8**, 29–37 (1928)
59. Rees, C. W., Bozicevich, J., Reardon, L. V., and Jones, F., *Am. J. Trop. Med.*, **22**, 581–86 (1942)
60. Bozicevich, J., Hoyem, H. M., and Walston, V. M., *Public Health Repts. (U.S.)*, **61**, 529–34 (1946)
61. Bozicevich, J., in *Parasitic Infections in Man*, 50–52 (Most, H., Ed., Columbia Univ. Press, New York, N.Y., 229 pp., 1951)
62. Terry, L. L., and Bozicevich, J., *Southern Med. J.*, **41**, 691–702 (1948)
63. Hussey, K. L., and Brown, H. W., *Am. J. Trop. Med.*, **30**, 147–54 (1950)
64. Most, H., Miller, J. W., and Grossman, E. J., *Am. J. Trop. Med.*, **30**, 491–97 (1950)
65. Tonney, F. O., Hoeft, G. L., and Spector, B. K., *J. Am. Med. Assoc.*, **101**, 1638–39 (1933)
66. Schoenleber, A. W., *J. Trop. Med. Hyg.*, **44**, 41–43 (1941)
67. Faust, E. C., *Amebiasis*, 110–13 (Charles C Thomas, Publisher, Springfield, Ill., 154 pp., 1954)
68. Anderson, H. H., Bostick, W. L., and Johnstone, H. G., *Amebiasis: Pathology, Diagnosis and Chemotherapy* (Charles C Thomas, Publisher, Springfield, Ill., 431 pp., 1953)
69. Imboden, C. A., in *Problems in Amebiasis*, 99–109 (Charles C Thomas, Publisher, Springfield, Ill., 119 pp., 1955)
70. Sodeman, W. A., and Beaver, P. C., *Am. J. Med.*, **12**, 440–46 (1952)
71. Sappenfield, R. W., Carter, F. R. N., Culbertson, C. G., Brooke, M. M., Payne, F. M., and Frye, W. W., *J. Am. Med. Assoc.*, **159**, 1009–12 (1955)
72. Craig, C. F., *Am. J. Trop. Med.*, **20**, 799–801 (1940)
73. Hauer, A., *Deut. Tropenmed. Z.*, **48**, 60–67 (1943)
74. Westphal, A., *Z. Hyg. Infektionskrankh.*, **128**, 56–72 (1948)
75. Berberian, D., Dennis, E., Korns, R. F., and Angelo, C., *J. Am. Med. Assoc.*, **148**, 700–4 (1952)
76. Hoekenga, M., *J. Lab. Clin. Med.*, **39**, 267–70 (1952)
77. Beaver, P. C., Jung, R. C., Sherman, H. J., Read, T. R., and Robinson, T. A., *Am. J. Trop. Med. Hyg.*, **5**, 1015–29 (1956)
78. Faust, E. C., and Russell, P. F., in *Clinical Parasitology*, 6th ed., 110–39 (Lea & Febiger, Philadelphia, Pa., 1078 pp., 1957)
79. Hoare, C. A., *Vet. Rev. Annot.*, **1**, 62–68 (1955)
80. Latyshev, N. I., Kojevnikov, P. V., and Povalishina, T. P., *State Pub. Med. Lit. Medgiz*, Moscow (Russian text, 1953), cited in Hoare, C. A., *Vet. Rev. Annot.*, **1**, 62–68 (1955)

81. Ansari, N., and Faghih, M., *Ann. parasitol. humaine et comparée*, **28**, 241–46 (1953)
82. del Ponte, E., *Bol. Ofic. Sanit. panam.*, **32**, 223–31 (1952)
83. Herrer, A., *Rev. Med. Exptl.* (Lima), **7**, 62–69 (1948)
84. Deane, L. M., and Deane, M. P., *O Hospital*, Rio de Janeiro, **45**, 419–21, 703–7 (1954)
85. Dias, E., and Chandler, A. C., *Mem. inst. Oswaldo Cruz*, **47**, 403–41 (1951)
86. Woody, N. C., and Woody, H. B., *J. Am. Med. Assoc.*, **159**, 476–77 (1955)
87. Pifano, F., *Arch venezolanos patol. trop. y parasitol. méd.*, **2**, 89–120 (1954)
88. Groot, H., Renjifo, S., and Uribe, C., *Am. J. Trop. Med.*, **31**, 673–91 (1951)
89. DeLeon, J. R., *Publs. inst. invest. cient. (Guatemala)*, No. **3**, 1–34 (1949)
90. Flock, H., y Abonnenc, C., *Bull. soc. pathol. exotique*, **42**, 394–400 (1949)
91. Groot, H., *Anales soc. biol. Bogotá*, **6**, 109–26 (1954)
92. Taylor, R. M., in *Yellow Fever*, 427–538 (McGraw-Hill Book Co., Inc., New York, N.Y., 710 pp., 1951)
93. Faust, E. C., and Russell, P. F., in *Clinical Parasitology*, 6th ed., 892–95 (Lea & Febiger, Philadelphia, Pa., 1078 pp., 1957)
94. Chang, P. C. H., *J. Parasitol.*, **42**, 126–36 (1956)
95. Das Gupta, N. N., Guha, A., and De, M. L., *Exptl. Cell Research*, **6**, 353–60 (1954)
96. Lofgren, R., *J. Bacteriol.*, **60**, 617–25 (1950)
97. Kleinschmidt, A., and Kinder, E., *Zentr. Bakteriol. Parasitenk., Abt.I*(b) *Orig.*, **156**, 219–24 (1950)
98. Kleinschmidt, A., and Schleich, F., *Z. Tropenmed. u Parasitol.*, **3**, 42–46 (1951)
99. Meyer, H., and Porter, K. R., *Parasitol.*, **44**, 16–23 (1954)
100. Von Brand, T., and Agosín, M., *J. Infectious Diseases*, **97**, 274–79 (1955)
101. Yaeger, R., *"Studies on the Isolation and* In Vitro *Metabolism of the Bloodstream and Intracellular Stages of* Trypanosome cruzi (Doctoral dissertation (Abstract), Tulane Univ., New Orleans, La., July 19, 1955)
102. Jenkins, A. R., and Grainge, E. B., *Trans. Roy. Soc. Trop. Med. Hyg.*, **50**, 481–84 (1956)
103. Pizzi, T., *Inmunología de la Enfermedad de Chagas.* (Univ. de Chile, Santiago, 183 pp., 1957)
104. Jacobs, L., *Ann. N.Y. Acad. Sci.*, **64**, 154–79 (1956)
105. Garnham, P. C. C., *Trans. Roy. Soc. Trop. Med. Hyg.*, **51**, 93–95 (1957)
106. Gustafson, P. V., Agar, H. D., and Cramer, D. I., *Am. J. Trop. Med. Hyg.*, **3**, 1008–21 (1954)
107. Fulton, J. D., and Spooner, D. F., *Trans. Roy. Soc. Trop. Med. Hyg.*, **51**, 123–24 (1957)
108. Jacobs, L., Melton, M. L., and Cook, M. K., *J. Parasitol.*, **41**, 353–61 (1955)
109. Beattie, C. P., *Trans. Roy. Soc. Trop. Med. Hyg.*, **51**, 96–103 (1955)
110. Siim, J. C., *Assoc. intern. Pédiat., 8th Congr.*, Summary, 13 (1956) cited in Beattie, C. P., *Trans. Roy. Soc. Trop. Med. Hyg.*, **51**, 96–103 (1957)
111. Sabin, A. B., and Feldman, H. A., *Science*, **108**, 660–63 (1948)
112. Sabin, A. B., *Pediatrics*, **4**, 443–53 (1949)
113. Cathie, I. A. B., *Trans. Roy. Soc. Trop. Med. Hyg.*, **51**, 104–10 (1957)

114. Jacobs, L., and Lunde, M. N., *Science*, **125**, 1035 (1957); *J. Parasitol.*, **43**, 308 (1957)
115. Hellbrugge, T., *Deut. Med. Wochschr.*, **74**, 385–89 (1949)
116. Prior, J. A., Cole, C. R., Docton, F. L., Saslaw, S., and Chamberlain, D. M., *Arch. Internal Med.*, **92**, 308–20 (1953)
117. Gibson, C. L., and Eyles, D. E., *Am. J. Trop. Med. Hyg.*, 990–1000 (1957)
118. Beverley, J. K. A., Beattie, C. P., and Roseman, C., *J. Hyg.*, **52**, 37–46 (1954)
119. Piekarski, G., in Beattie, C. P., *Trans. Roy. Soc. Trop. Med. Hyg.*, **51**, 96–103 (1957)
120. Harboe, A., *Acta Pathol. Microbiol. Scand.*, **93**, 325–30 (1952)
121. Feldman, H. A., *Assoc. intern. Pédiat., 8th Congr.*, in Beattie, C. P., *Trans. Roy. Soc. Trop. Med. Hyg.*, **51**, 96–103 (1957)
122. Olafson, P., and Monlux, W. S., *Cornell Vet.*, **32**, 176–90 (1942)
123. Siim, J. C., *J. Am. Med. Assoc.*, **147**, 1641 (1951)
124. Havlík, O., *Časopis lékářu českých.*, **90**, 1516–18 (1951); cited in Jacobs, L., *Ann. N.Y. Acad. Sci.*, **64**, 154–79 (1956)
125. Woke, P. A., Jacobs, L., Jones, F. E., and Melton, M. L., *J. Parasitol.*, **39**, 523–32 (1953)
126. Frenkel, J. F., *Am. J. Trop. Med. Hyg.*, **2**, 390–411 (1953)
127. Ash, J. E., and Spitz, S., *Pathology of Tropical Diseases. An Atlas*, 197 (W. B. Saunders Co., Philadelphia, Pa., 350 pp., 1945)
128. Eyles, D. E., *Am. J. Trop. Med. Hyg.*, **2**, 429–44 (1953)
129. Brown, J., and Jacobs, L., *Ann. Internal Med.*, **44**, 565–72 (1956)
130. Ammich, O., *Arch. pathol. Anat. u. Physiol. Virchow's*, **302**, 539–54 (1938)
131. Van der Meer, G., and Brug, S. L., *Ann. Soc. belge méd. trop.*, **22**, 301–7 (1942)
132. Wyatt, J. R., Simon, T., Trumball, M. L., and Evans, M., *Am. J. Clin. Pathol.*, **23**, 353–62 (1953)
133. Baar, H. S., *J. Clin., Pathol.*, **8**, 19–24 (1955)
134. Pizzi, T., and Díaz, M., *Rev. chilena pediat. (Santiago, Chile)*, **27**, 281–99 (1956)
135. Vanek, J., and Jírovek, O., *Zentr. Bakteriol., Parasitenk., Abt.I*(b) *Orig.*, **158**, 120–27 (1952)
136. Westphal, A., *Z. Tropenmed. u. Parasitol.*, **4**, 549–54 (1953)
137. Lunseth, J., Kirmse, T., Prezyna, A., and Gerth, R., *J. Pediat.*, **46**, 137–45 (1955)
138. Bustamente, W., Moreno, L., Doberti, A., and Román, C., *Rev. chilena pediat. (Santiago, Chile)*, **27**, 281–93 (1956)
139. Hallman, N., and Ylppö, A., *Acta Paediat. Suppl.*, **100**, 382–96 (1954)
140. Pillemer, L., Blum, L., Lepow, I. H., Ross, O. A., Todd, W., and Wardlaw, A. C., *Science*, **120**, 279 (1954); *J. Exptl. Med.*, **103**, 1–13 (1956)
141. News briefs, *Bol. chileno de Parasitol. (Santiago, Chile)*, **11**, 73 (1956)
142. Garnham, P. C. C., *Ann. Rev. Microbiol.*, **8**, 153–66 (1954)
143. Maegraith, B., Jones, E. S., and Andrews, W. H. H., *Trans. Roy. Soc. Trop. Med. Hyg.*, **45**, 15–44 (1951)
144. Allison, A. C., *Exptl. Parasitol.*, **6**, 418–47 (1957)
145. Faust, E. C., and Russell, P. F., *Clinical Parasitology*, 6th ed., 283 (Lea & Febiger, Philadelphia, Pa., 1078 pp., 1957)

146. Coatney, G. R., in *Parasitic Infections in Man,* 187–202 (Most, H., Ed., Columbia University, New York, N.Y., 229 pp., 1951)
147. Council on Pharmacy & Chemistry, Am. Med. Assoc., *J. Am. Med. Assoc.,* **137,** 1532 (1948)
148. Laing, A. B. G., *Trans. Roy. Soc. Trop. Med. Hyg.,* **50,** 496–504 (1956)
149. Hernández, T., Myatt, A. V., Coatney, G. R., and Jeffery, G. M., *Am. J. Trop. Med. Hyg.,* **2,** 797–804 (1953)
150. Arnold, G., Alving, A. S., Hockwald, R. S., Clayman, C. B., Dern, R. J., Bentler, E., and Jeffery, G. M., *J. Lab. Clin. Med.,* **44,** 429–38 (1954)
151. Russell, P. F., *Trop. Med. & Hyg. News,* **6,** 9 (1957)
152. Livadas, G. A., and Georgopoulos, G., *Bull. World Health Organization,* **8,** 497–511 (1953)
153. Trápido, H., *Am. J. Trop. Med. Hyg.,* **1,** 853–61 (1952)
154. Busvine, J. R., *Trans. Roy. Soc. Trop. Med. Hyg.,* 11–31 (1957)
155. Faust, E. C., *Animal Agents and Vectors of Human Disease,* 273–79 (Lea & Febiger, Philadelphia, Pa., 660 pp., 1955)
156. Kirby-Smith, J. L., Dove, W. E., and White, G. F., *Arch. Dermatol. and Syphilol.,* **13,** 137–73 (1926)
157. Wilson, J. F., *Southern Med. J.,* **45,** 127–31 (1952)
158. Knox, J. M., *J. Louisiana State Med. Soc.,* **105,** 69–72 (1953)
159. Maplestone, P. A., *Indiana Med. Gaz.,* **68,** 251–57 (1933)
160. Hunter, G. W., III, and Worth, C. B., *J. Parasitol.,* **31,** 366–72 (1945)
161. Schenone, H., Robles, E., and Montoya, C., *Bol. chileno parasitol.,* **11,** 71–73 (1956)
162. Fülleborn, F., *Bernhard Nocht Festschrift, Hamburg Univ. Abhandl. Geb. Auslandsk.,* **26,** 121–33 (1927)
163. Mayhew, R. L., *Proc. Soc. Exptl. Biol Med.,* **66,** 12–14 (1947)
164. Beaver, P. C., *Exptl. Parasitol.,* **5,** 587–621 (1956)
165. Faust, E. C., *Rev. de Parasitol.* (Habana), **2,** 315–41 (1936)
166. Faust, E. C., and De Groat, A., *Am. J. Trop. Med.,* **20,** 350–75 (1940)
167. Fülleborn, F., *Arch. Schiffs- u. Tropen-Hyg.,* **30,** 721–32 (1926)
168. Caplan, J. P., *Brit. Med. J.,* **I,** 396 (1949)
169. Napier, L. E., *J. Trop. Med. Hyg.,* **52,** 46–48 (1949)
170. Perlingiero, J., and György, P., *Am. J. Diseases Children,* **78,** 153–81 (1949)
171. Zuelzer, W., and Apt, L., *Am. J. Disease Children,* **73,** 34–43 (1947)
172. Mercer, R., Lund, H., Bloomfield, R., and Caldwell, R., *Am. J. Diseases Children,* **80,** 46–58 (1950)
173. Wilder, H. C., *Trans. Am. Acad. Ophthalmol. Otolaryngol.,* **55,** 99–100 (1950)
174. Behrer, M. R., *J. Pediat.,* **38,** 635–40 (1951)
175. Beautyman, W., and Woolf, A., *J. Pathol. Bacteriol.,* **63,** 635–47 (1951)
176. Beaver, P. C., Snyder, H., Carrera, G. M., Dent, J. H., and Lafferty, J. W., *Pediatrics,* **9,** 7–19 (1952)
177. Nichols, R. L., *J. Parasitol.,* **42,** 349–62 (1956)
178. Nichols, R. L., *J. Parasitol.,* **42,** 363–82 (1956)
179. De Magalhães, P. S., *Rev. curs. prat. theor. Fac. Med., Rio de Janeiro,* **3,** 129–216 (1887)
180. Faust, E. C., Thomas, E. P., and Jones, J., *J. Parasitol.,* **27,** 115–22 (1941)

181. Teixeira de Freitas, J. F., and Mayall, R., *Rev. brazil. med. (Rio de Janeiro)*, **10**, 463–67 (1953)
182. Skrjabin, K. I., Al'tgauzen, A. J., and Schul'man, E. S., *Trop. Med. i. Veterin. Moskva*, **8**, 9–11 (1930)
183. Kotlán, S., *Acta Vet. Acad. Sci. Hung.*, **1**, 69–79 (1951)
184. Dubini (1850) cited in *Brumpt, E., Précis de Parasitologie*, **I**, 928 (Masson & Cie, Paris, France, 1949)
185. Bevacqua, R., and Wirth, A., *Ann. parasitol. humaine et comparée*, **25** 37–41 (1950)
186. Desportes, C., *Ann. parasitol. humaine et comparée*, **17**, 380–404 (1939); **17**, 515–32 (1940)
187. Addario, C., *Ann. ottalmol. e clin. oculist.*, **14**, 135–48 (1885)
188. Parodi, S. E., and Bonavia, L. L., *Semana méd. (Buenos Aires)*, **27**, 543–48 (1920)
189. Faust, E. C., *Z. Tropenmed. u. Parasitol.*, **8**, 60–68 (1957)
190. Faust, E. C., Agosín, M., Garcia-Laverde, A., Sayad, W. Y., Johnson, V. M., and Murray, N. A., *Am. J. Trop. Med. Hyg.*, **1**, 239–49 (1952)
191. Beaver, P. C. (Personal communication, 1957)
192. Thomas, L. J., *Proc. Helminthol. Soc. Wash., D.C.*, **19**, 124–26 (1952)
193. Feng, L. C., Tung, M., and Su, S., *Chinese Med. J.*, **73**, 149–62 (1955)
194. Singh, S., and Rao, V., *J. Helminthol.*, **28**, 155–58 (1954)
195. Sen, K., and Ghose, N., *Brit. J. Ophthalmol.*, **29**, 618–26 (1945)
196. Prommas, C., and Daengsvang, S., *J. Parasitol.*, **22**, 180–86 (1936)
197. Daengsvang, S., *J. Parasitol.*, **35**, 116–21 (1949)
198. Prijyanonda, B., Pradatsundarasar, A., and Viranuvatti, V., *Ann. Trop. Med. Parasitol.*, **49**, 121–22 (1955)
199. Lie, K. J. (Personal communication, 1949)
200. Toumanoff, C., and Le-Van Phung, *Bull. Soc. Pathol. exotique*, **40**, 168–74 (1947)
201. Chang, E., *Chinese Med. J.*, **67**, 166–68 (1949)
202. Sison, A., and Florentin, A., *Acta Med. Philippina*, **7**, 231–38 (1951)
203. Yogore, M., and Juliano, S., *Acta Med. Phillippina*, **7**, 239–45 (1951)
204. Miyazaki, I., *Kyushu Mem. Med. Sci.*, **5**, 13-27 (1954)
205. Nagao, M., *Fukuoka-Igaki-Zassi*, **46**, 2013–20 (1955)
206. Witenberg, G., Jacoby, J., and Steckelmacher, S., *Ophthalmologia*, **119**, 114–22 (1950)
207. Price, D. L., and Jachowski, L. A., *J. Parasitol.*, **41**, 46 (1955)
208. Jachowski, L. A., Jr., *Naval Med. Research Inst. Lecture and Rev. Ser.* No. 55, **7**, 345–59 (1955)
209. Gordon, R. M., *Trans. Roy. Soc. Trop. Med. Hyg.*, **49**, 97–105 (1955)
210. Kershaw, W. E., *Trans. Roy. Soc. Trop. Med. Hyg.*, **49**, 143–50 (1955)
211. Duke, B. O. L., *Trans. Roy. Soc. Trop Med. Hyg.*, **49**, 115–21 (1955)
212. Peel, E., and Chardome, M., *Ann. soc. belge méd. trop.*, **26**, 117–56 (1946)
213. Chardome, M., and Peel, E., *Ann. soc. belge méd. trop.*, **31**, 571–77 (1951)
214. Weinstein, P. P., and Jones, M. F., *J. Parasitol.*, **42**, 215–36 (1956)
215. Lawrence, J. J., *Australian J. Biol. Med. Sci.*, **26**, 1–8 (1948)
216. Weinstein, P. P., *J. Parasitol.*, **35**, 14 (1949)
217. Weinstein, P. P., *Am. J. Hyg.*, **58**, 352–76 (1953)

218. Weinstein, P. P., *J. Parasitol.*, **40**, 14–15 (1954)
219. Weller, T. H., *Am. J. Pathol.*, **9**, 503–15 (1943)
220. Pitts, T. D., and Ball, G. H., *J. Parasitol.*, **41**, 47–48 (1955)
221. Faust, E. C., *Anais inst. med. trop.* (Lisbôa), **9**, 1037–57 (1952)
222. Khalil, M., and Azim, M. A., *J. Egypt. Med. Assoc.*, **18**, 371–77 (1935)
223. Meira, J. A., *Bull. World Health Organization*, **2**, 31–37 (1949)
224. Hsü, H. F., *Am. J. Trop. Med.*, **30**, 397–410 (1950)
225. Le Gac, P., Lemaigre, C., and Tournier-Lasserve, C., *Bull. Soc. Pathol. exotique*, **46**, 685–88 (1953)
226. Kuntz, R. E., *Proc. Helmithol. Soc. Wash., D.C.*, **19**, 123–24 (1952)
227. Barbosa, F. S. Dobbins, J. E., Jr., and Vasconcelos, C. M., *Publ. Avulsas inst. Aggeu Magalhães (Recife, Brazil)*, **2**, 63–67 (1953)
228. Pimental, J., *Rev. brasil. malariol. doenças trop.*, **5**, 219–22 (1953)
229. Schwetz, J., *Trans. Roy. Soc. Trop. Med. Hyg.*, **50**, 275–82 (1956)
230. Kuntz, R. E., and Malakatis, G. M., *Exptl. Parasitol.*, **4**, 1–20 (1955)
231. Kuntz, R. E., and Malakatis, G. M., *Am. J. Trop. Med. Hyg.*, **4**, 75–89 (1955)
232. Kuntz, R. E., *Am. J. Trop. Med. Hyg.*, **4**, 383–413 (1955)
233. Hsü, H. F., and Hsü, S. Y. Li, *Am. J. Trop. Med. Hyg.*, **5**, 521–28 (1956)
234. Hsü, H. F., and Hsü, S. Y. Li, *Am. J. Trop. Med. Hyg.*, **5**, 136–44 (1956)
235. Cowper, S. G., *Ann. Trop. Med. Parasitol.*, **41**, 173–77 (1947)
236. Files, V. S., *Parasitol.*, **41**, 264–69 (1951)
237. Newton, W. L., *Exptl. Parasitol.*, **2**, 242–57 (1953)
238. Barbosa, F. S., Coelho, M. V., and Carneiro, E., *Trans. Roy. Soc. Trop. Med. Hyg.*, **50**, 296–97 (1956)
239. Vogel, H., *Ann. Trop. Med. Parasitol.*, **41**, 266–77 (1947)
240. Short, R. B., *J. Parasitol.*, **34**, 240–42 (1948)
241. Gönnert, R., *Z. Tropenmed. u. Parasitol.*, **1**, 272–79 (1949)
242. Short, R. B., *J. Parasitol.*, **37**, 547–55 (1951)
243. Fairley, N. H., *Trans. Roy. Soc. Med. Hyg.*, **45**, 279–303 (1951)
244. Oliver-González, J., Bauman, P. M., and Benenson, A. S., *Am. J. Trop. Med. Hyg.*, **4**, 443–52 (1955)
245. Papirmeister, B., and Bang, F. B., *Am. J. Hyg.*, **48**, 74–80 (1948)
246. Vogel, H., and Minning, W., *Zentr. Bakteriol., Parasitenk., Abt.I*(b), *Orig.*, **153**, 99–105 (1948)
247. Stirewalt, M. A., and Evans, A. S., *Exptl. Parasitol.*, **4**, 123–42 (1955)
248. Kagan, I. G., *Exptl. Parasitol.*, **4**, 361–76 (1955)
249. McMullen, D. B., *Am. J. Trop. Med. Hyg.*, **1**, 671–79 (1952)
250. Ritchie, L. R., *Am. J. Trop. Med. Hyg.*, **4**, 426–41 (1955)
251. Pesigan, T. P., and Masilungan, V. A., *J. Philippine Med. Assoc.*, **26**, 17–30 (1950)
252. McMullen, D. B., Hubendick, B., Pesigan, T. P., and Bierstein, P., *J. Parasitol.*, **39**, 17 (1953)
253. Kuntz, R. E., and Wells, W. H., *Am. J. Trop. Med.*, **31**, 784–824 (1951)
254. Olivier, L., *Am. J. Trop. Med. Hyg.*, **4**, 415–23 (1955)
255. Vaughn, C. M., Olivier, L., Hendricks, J. R., and Mackie, T. T., *Am. J. Trop. Med. Hyg.*, **3**, 518–28 (1954)
256. Meleney, H. E., *Am. J. Trop. Med. Hyg.*, **3**, 209–18 (1954)
257. Cort, W. W., *J. Am. Med. Assoc.*, **90**, 1027–29 (1928)

258. McMullen, D. B., and Beaver, P. C., *Am. J. Hyg.*, **42**, 128–54 (1954)
259. Hunter, G. W., III, Ritchie, L. S., and Tanabe, H., *Trans. Roy. Trop. Med. Hyg.*, **45**, 103–12 (1951)
260. Macfarlane, W. V., *Am. J. Hyg.*, **50**, 143–67 (1949)
261. Szidat, L., *Comun. inst. nac. invest. cienc. nat. museo argentino cienc. nat. "Bernardino Rivadavia,"* **2**, 129–50 (1951)
262. Stunkard, H. W., Hinchliffe, M. C., *J. Parasitol.*, **38**, 248–65 (1952)
263. Chu, G. W. T. C., *Science*, **115**, 151–53 (1952)
264. Penner, L. R., *J. Parasitol.*, **39**, 20 (1953)
265. Buckley, J. J. C., *J. Helminthol.*, **16**, 117–20 (1938)
266. Alves, W., *Trans. Roy. Soc. Trop. Med. Hyg.*, **47**, 272 (1953)
267. Cort, W. W., *Am. J. Hyg.*, **52**, 251–307 (1950)
268. Olivier, L., *Am. J. Hyg.*, **49**, 290–301 (1949)
269. Hsü, H. F., and Ameel, D. J., *Am. J. Trop. Med. Hyg.*, **5**, 841–46 (1956)
270. Cameron, T. W. M., *Proc. Roy. Soc. Med. (Sec. Trop. Diseases, Parasitol.)*, **20**, 302–11 (1927)
271. Rausch, R., and Schiller, E. L., *Science*, **113**, 57–58 (1951)
272. Rausch, R., *Am. J. Trop. Med. Hyg.*, **5**, 1086–92 (1956)
273. Thomas, L. J., Babero, B. B., Gallicchio, V., and Lacey, R. J., *Science*, **120**, 1102–3 (1954)
274. Vogel, H., *Deut. Med. Wochschr.*, **80**, 931–32 (1955)
275. Vogel, H., *Z. Tropenmed. u. Parasitol.*, **8**, 404–56 (1957)
276. Mankau, S. K., *J. Parasitol.*, **43**, 153–57 (1957)
277. Thomas, L. J., and Babero, B. B., *J. Parasitol.*, **42**, 500 (1956)

# THE CONTROL AND ERADICATION OF ANIMAL DISEASES IN THE UNITED STATES[1]

By William A. Hagan
*New York State Veterinary College, Cornell University, Ithaca, New York*

## Introduction

Somewhere in prehistoric times, man learned to tame and domesticate animals to serve his purposes. Apparently, the dog was the first of the series and long afterward came the horse. Only after man had given up his nomadic habits and settled down to agrarian pursuits did the cow, the pig, the sheep, the goat, and various kinds of poultry join the group. All of these had been domesticated by the time the recording of history began. Since animals often represented his most valued possessions, it is not surprising that early man developed a keen interest in their health and well-being. This interest has continued and, with increasing population, the control or eradication of animal diseases has become a very important economic and health problem.

## The Epizootiology of Animal Diseases

As they are in man, the infectious diseases of animals are transmitted most often by more or less direct contact between sick and well individuals. A few highly contagious maladies are transmitted with some frequency by contaminated objects (e.g., foot-and-mouth disease), but most diseases travel from place to place no faster than animals travel. Since the individual animal does not have the same importance as the human individual, the animal disease-control official has a great advantage over his colleagues, who are engaged in the same pursuit with reference to man, in that he can destroy diseased animals to prevent their spreading the infection. Also, he can control the movements and contacts of diseased animals much more easily than the health officer can control human subjects. A man with tuberculosis, for example, generally finds little to prevent his travelling wherever he pleases and making as many contacts with susceptible people as he wishes. A tuberculous cow, on the other hand, in most enlightened countries and as soon as she is recognized to be affected, cannot be moved from the premises of her owner for any purpose except to go to a slaughtering establishment. In these respects animal disease control is easier than control of human diseases.

On the other hand, there are other factors which make control of animal

[1] The survey of the literature pertaining to this review was concluded in December, 1957.

diseases more difficult than control of those of man. Domestic animals do not have the sanitary habits that characterize civilized man. Most animals deposit their excretions more or less indiscriminately, frequently contaminating their own feed and that of their herd or flock mates. This is especially true when animals are forced to live within buildings or on small areas of soil. Their human masters often provide poor living conditions thus depriving them of the chance to exercise some of the small sanitary instincts that they have. The man who is ill generally is aware of his ill-health, usually takes steps to procure advice and treatment, and often takes precautions to protect others who associate with him. The sick animal often is not recognized to be ill until its disease is far advanced and it may have passed its infection along to many others. Even though the owner recognizes that an animal is not well, some do nothing about it and others vainly try all sorts of home remedies before calling in expert advice. These actions, of course, allow diseases to spread.

Farm owners have little sentimental attachment for their animals; whether they are healthy or unhealthy, or even whether they live or die is almost wholly an economic matter. Instances could be cited in which owners have deliberately accepted animal-disease losses which they could have prevented, because it was judged to be more profitable to accept them than to incur the expense of preventive measures. The more progressive farmers recognize that a high order of health and vigor is necessary for a high level of production. They use care in selecting their original stock, take precautions to avoid, so far as possible, the purchase of diseased animals, endeavor to protect their herds and flocks from exposure to infection from outside, and use veterinary service to help them attain these ends. Less progressive or poorly informed owners use less care in all these respects. Many animal owners use almost no precautions to protect against disease except those which are forced on them by laws and regulations of their government.

In this country, to a greater degree than in any other, animals have become travellers. Whereas, a century ago only a very few animals travelled farther than they could walk, today all kinds of animals travel daily by railway, motor trucks, and even by planes. Simms (1) has pointed out that it took 50 years for tick-fever to travel northward across the State of North Carolina. This occurred before the days of rapid transportation, hence the disease travelled no faster than cows were moved on foot. Commenting on this situation, Simms once observed that "sick cows do not walk very far." Today thousands of animals travel much greater distances every day, and unfortunately, in spite of many precautions, some of these animals are diseased and serve to disseminate their maladies. Three outbreaks of foot-and-mouth disease occurred in the United States before 1900. All were in the northeastern states. All three died out without much assistance from man largely because heavy winter snows immobilized cattle and, to a large ex-

tent, even their human attendants. In the six outbreaks that have occurred more recently, the disease has spread rapidly and far in spite of the strenuous efforts of disease-control officials to stop them. Traffic in cattle, sometimes swine, has been responsible for all of these spreads.

### Laws and Regulations for the Control of Animal Diseases in the United States

The control of animal diseases within the individual states is wholly a state function. The Federal government is concerned with the subject only as a result of the authority conferred by the Constitution (Article I, Sect. 8, paragraph 3) which gives Congress the power, "To regulate commerce with foreign Nations, and among the several States, and with the Indian tribes." Although within its borders each state is supreme and may enact and enforce such laws and make regulations for the control of diseases of livestock as it sees fit, the Federal government assumes jurisdiction, under its constitutional power, when animals are shipped from one state to another, or from any state to any foreign country. The Federal government may not interfere with the shipping of livestock, as a disease-control measure, so long as it is confined to the territory of any single state. It may, however, quarantine a part of any state, or entire states, to prevent shipping from these areas to outside areas. The Federal government has exercised its authority, under the interstate commerce clause, for many years to prevent the shipping of diseased animals. All that enter into interstate trade are required to be accompanied by health certificates, issued by qualified and licensed veterinarians, indicating that the individuals have been given physical examinations and found to be healthy. These certificates also serve to prove that the animal, if it is of the bovine species, has been subjected to a tuberculin test and generally also a blood test for brucellosis, and been found negative to both tests. Inspectors at strategic points along the rail lines check these certificates which are attached to the weighbills and accompany the cattle on their journey. These measures of the Federal government complement those of the states. Unquestionably they have done much to reduce the spread of disease.

### Animal Disease Eradication in the United States

From time to time the Federal government, through its Department of Agriculture, has assumed leadership in nationwide programs directed toward control and eradication of a number of animal diseases. These, of necessity, are cooperative projects with the individual states, since the states cannot be forced into them. For such programs uniform rules generally are worked out in advance, after which agreements are signed between Federal authorities and such states as wish to cooperate. Generally, the cost has been shared. In the beginning of most of these programs not all states have

signed agreements immediately. The work has been begun in those states which have signed agreements and others have been included upon their decision to cooperate. In all the programs so far, all states eventually have cooperated. Uncooperative states generally are forced by economic considerations to join in the effort when a majority of states are already in the program. A feature of these programs has been the policy of paying indemnity to owners for animals which are sacrificed in the course of the work. This action has been justified on the basis that owners of such animals seldom are wholly at fault for having diseased animals, and that it is in the public good to destroy them in order to achieve the desired goal. By such programs it has been possible to eradicate several important diseases that either were already very costly or promised to become very costly if allowed to spread unchecked. Control, without achieving eradication, has been the result in some instances, but these programs are not yet finished and eradication is the ultimate goal. A brief account of the principal eradication programs follows.

*Contagious pleuropneumonia of cattle.*—Pleuropneumonia is a very destructive disease in Asia and Africa. It is caused by a minute bacillus which produces a highly fatal, chronic pneumonia in cattle. At one time it was nearly worldwide, but today the range of the disease has been greatly reduced. When pleuropneumonia invaded South Africa in 1854, it is said to have killed more than one hundred thousand animals within two years (2). On many occasions it has invaded western Europe and during one of these invasions, in 1843, it found its way to New York in a ship's cow (3). Little or nothing was done to control the disease for 40 years, with the notable exception that the State of Massachusetts managed to stamp it out within its borders in 1866. By 1880, the disease had been recognized in New York, Massachusetts, Pennsylvania, New Jersey, Delaware, Maryland, West Virginia, Connecticut, and the District of Columbia. Since the flow of cattle was principally from west to east the disease was not found in any of the western states until 1885 when it appeared in Kentucky, Illinois, and Missouri (3). The western spread posed a great threat to the great beef herds that roamed the fenceless plains, from which it would have been very difficult to eradicate had a firm foothold been gained. The interest of Congress was finally aroused, and it was because of this interest that the Bureau of Animal Industry was founded. The newly created agency was directly charged with the marshalling of all available facilities for eradicating this disease. A campaign against the disease was immediately launched by the Federal agency working in conjunction with the forces of the states where the disease existed. Infected herds were hunted down and destroyed and the owners compensated for their losses. On September 6, 1892, the Secretary of Agriculture issued a proclamation declaring the country to be free from bovine contagious pleuropneumonia. Five years and about one

and one-half millions dollars had been spent in the task. The job had been well done since the disease has never reappeared in North America.

*Glanders in horses.*—Glanders is a chronic bacterial disease of animals of the equine species. Certain cats are susceptible and also man, but these species seldom play a part in the natural history of the disease. Glanders was recognized in the days of the ancient Roman Empire. Until recent times the disease was found in nearly all parts of the world where there were concentrations of horses. During the nineteenth century the principal centers of glanders were in the great cities, in the huge stables of the horse-car companies, and the great trucking concerns which often housed thousands of horses under a single roof. Military organizations also were plagued with glanders.

Evidently glanders was imported from Europe very early since the disease was widespread in many of the northern cities of the United States when it was first recognized. The southern states were relatively free from this disease until the Civil War when it was introduced by the military operations. After the Civil War there was a wide distribution of the disease throughout the rural areas of the entire country by horses returned from military service. Rural areas also were frequently infected by city horses that were retired to lighter farm work after outliving their usefulness for heavy city work.

The newly established Bureau of Animal Industry made the first efforts to combat glanders—in the District of Columbia in 1888. Since there were no remedies for the disease, the effort took the form of finding and destroying all infected animals. Since infected animals often became spreaders of infection before signs of the disease became apparent, methods of early diagnosis were necessary for success. Mallein, a product analogous to tuberculin but prepared from the metabolic products of the organism of glanders, came into use in 1895. The complement-fixation test, first used for the diagnosis of glanders in Europe in 1909, was introduced into the United States in 1911 (4). By widespread use of these tests on a compulsory basis, especially in the large cities where the great centers of infection existed, the incidence of the disease was rapidly reduced. The replacement of horsecars with electric streetcars, and later the replacement of essentially all work horses by automobiles and motor trucks, certainly greatly aided the eradication work; however, it is clear that the work was far along before these influences became fully effective. Since 1920 very few cases of glanders have been found in the United States. At the present time the disease appears to have been eradicated.

*Foot-and-mouth disease.*—The only large land masses which are free from foot-and-mouth disease today are Australia and North America. These regions have been able to remain free partly because of their relative isolation but largely because of their rigidly enforced laws and regulations

relating to importation of susceptible animals and animal products from regions where the disease is known to exist.

Foot-and-mouth disease is caused by a virus, of which there are at least seven principal types and many subtypes. The diseases caused by the different types are clinically indistinguishable from each other. Since the different types do not cross-immunize, animals affected with one type may almost immediately after recovery demonstrate practically identical symptoms as a result of infection with a different type. Immunity, even to the same type, is relatively short-lived. Even though we now have some rather effective vaccines for foot-and-mouth disease, they have limited application. It is impracticable to immunize animals in advance of an outbreak because of the shortness of the period of protection, and especially because it is impossible to know what type of virus must be combatted. Vaccines are generally used during outbreaks to limit their spread in many European countries. A vaccine was used on a large scale in Mexico to combat the great outbreak which occurred there between 1946 and 1954.

The virus of foot-and-mouth disease is one of the most contagious agents known. In contagiousness it is exceeded only by the virus of human influenza. Since all secretions and excretions of affected animals contain virus, the premises where the disease occurs are extensively contaminated, and movements of animals, man, or vehicles of any kind are likely to spread the disease. It has been found that the most rigid of quarantine measures often fail to prevent the spread of virus. It is for this reason that countries that have some measure of isolation usually endeavor to stamp out outbreaks by quickly slaughtering and burying all susceptible animals living on premises where the disease is discovered. This policy is regularly used in the British Isles, and it has been the policy of this country and Canada in dealing with their isolated outbreaks. It was followed in Mexico until the Mexican government was forced by rebellious citizens to adopt a less drastic policy.

Foot-and-mouth disease is known to have gained a foothold in this country on at least nine different occasions. Three of these, in 1870, 1880, and 1884, apparently were caused by the importation of infected cattle from Europe. The later outbreaks, those of 1902, 1904, 1914, 1924, and 1929, quite certainly did not arise from the importation of live animals but rather in imported inanimate materials which contained virus. We had virtually no quarantine laws to protect us from infection at the time of the three early outbreaks. The laws which came later apparently have served well to protest us from imported diseased livestock, but it is almost impossible to provide protection from virus which may persist on contaminated objects. The Canadian outbreak of 1951 to 1952 is thought to have been started from virus contained on clothing of a recent immigrant from Europe.

It has been said before that the early outbreaks—those before 1900—subsided largely of their own accord, aided by severe winters and limited

movement of farm animals and farm people. Those of the twentieth century have been quite different; all have shown great contagiousness. They have spread quickly and sometimes widely. There is every evidence that the spread was caused by shipment of livestock over long distances, and through the movement of animals, people, and animal products, over shorter distances. In the 1902 outbreak which occurred in the New England states, the drastic method of control, that is, the immediate slaughter and burial of all infected and exposed animals followed by thorough cleaning and disinfecting of the premises, was used for the first time. Such premises are held in quarantine, and no susceptible livestock may be brought onto the premises for 30 days. Partial restocking is allowed at this time, and these animals are examined carefully every few days by veterinary inspectors. The quarantine is lifted and full stocking is permitted at the end of 90 days, providing no evidence of the disease appears on the immediate premises and on neighboring farms. In the various outbreaks, the disease has reappeared on a few premises, presumably from persisting virus, following this procedure, but generally there have been no subsequent cases.

The slaughter method has been challenged each time it has been used as being too drastic and wasteful. Experience has amply demonstrated, however, that it is the cheapest in the end. Half thorough measures, often used in continental European countries, too frequently fail. Compared with the values of the great industries which are at stake and the huge losses to be incurred should this disease ever become firmly established in this country, the cost of eradicating outbreaks has been very low.

The foot-and-mouth disease outbreak of 1908 was shown by Mohler and Rosenau to have originated in contaminated vaccine virus imported by an American manufacturing company from Japan, and there are strong suspicions that the 1902 outbreak may have originated in the same way. The greatest outbreak was that of 1914 which, before it was finally stamped out almost two years after it was first detected, had spread to 22 states and the District of Columbia. More than 3500 herds were found to harbor infected animals, more than 170,000 animals were destroyed and buried, and the cost was in excess of six million dollars. The origin of this outbreak was never satisfactorily traced. It apparently began in southwestern Michigan not far from Chicago, Illinois. Straw, in which imported tulip bulbs from Holland had been packed, was suspected.

In 1924, two outbreaks of foot-and-mouth disease occurred in the United States, one in California and another in Texas. No apparent connection between these outbreaks was discovered. The California outbreak was traced to swine herds that had been fed garbage from ships docking at the port of San Francisco. It was believed that the virus was brought ashore in this material which contained meat-scraps and trimmings from carcasses taken aboard in South American and Asiatic ports located in infected regions.

The source of the Texas outbreak was not traced. In the spring of 1925 another small outbreak occurred near Houston, Texas, in the area where the disease had occurred the previous year. This outbreak probably resulted from persisting virus.

During the 1924 outbreak in California, the disease appeared in the Stanislaus National Forest in wild deer. This was the first and only occasion when wild animals became involved in any outbreaks in this country. Professional hunters were sent into the forest, which was closed off to all other persons, and as many of the deer as could be found were destroyed. Early during this effort many deer which showed lesions of foot-and-mouth disease were shot, but as the winter progressed and the deer became scarcer, fewer and fewer infected animals were found. Not all animals were destroyed by any means, for within a few years the region was amply repopulated by the survivors; nevertheless the disease was stamped out.

The last outbreak of foot-and-mouth disease in the United States occurred in California in 1929. Again, ship's garbage was incriminated. This outbreak was stamped out with the usual methods within a short time. The two experiences with ship's garbage as a source of outbreaks resulted in the promulgation of regulations by the Secretary of Agriculture which now prohibits the practice of landing garbage from ships which have touched foreign ports in countries where foot-and-mouth disease is known to exist.

Several of the outbreaks of foot-and-mouth disease that have occurred in this country were well started before a definitive diagnosis was made. This delay results from the fact that there are several diseases here which clinically resemble it, except for its extreme contagiousness. When encountering the disease in regions which seem rather remote from foreign countries where foot-and-mouth disease occurs, veterinarians quite naturally are loathe to believe that it can be the dreaded exotic disease and are inclined to regard it as one of the more benign local maladies. Even state and Federal disease-control officials have been led astray on the diagnosis until they became aware the disease was spreading rapidly. Often, by the time the real nature of the disease was suspected or known, contacts have been shipped widely and many new centers of infection established. Since a diagnostic error is apt to be very costly, the Federal disease-control officials have been active in recent years in educating practicing veterinarians, state officials, and its own field men to the importance of regarding every animal showing suspicious symptoms as a potential case of foot-and-mouth disease. They are urged to report promptly all such cases, which are quickly investigated by trained workers.

Until quite recently the only way to make a definitive diagnosis of foot-and-mouth disease in the very beginning of an outbreak before the epizootiologic features of the disease had been observed, was by animal inoculation

tests. Fresh material from active lesions was collected and injected into one or more horses, cattle, and guinea pigs. The horses and cattle were inoculated in the epithelium of the superior surface of the tongue (intradermal-lingual), and the guinea pigs in the epithelial layer of the footpads of the hind feet. Foot-and-mouth disease virus will cause the formation of vesicles at the site of inoculation and secondary lesions later in cattle, but there will be no lesions in horses. The most common disease that may be confused with foot-and-mouth disease is vesicular stomatitis, another virus disease. The virus of vesicular stomatitis regularly causes lesions in horses, and may be distinguished in this way. The viruses of both diseases cause vesiculation in the footpads of the guinea pig, hence this animal does not assist in the differentiation. However, it does serve as a medium in which the virus may be maintained for confirming tests should the original tests prove indecisive.

In recent years the complement-fixation test has been so refined that it can be used not only to distinguish between the virus of foot-and-mouth disease and other viruses, but it is also capable of determining the types of foot-and-mouth disease virus (5). Prior to 1956 there were no laboratories in North America where such determinations could be made, since our laws would not permit any work with foot-and-mouth disease virus. The Plum Island Animal Disease Laboratory is now equipped to type the vesicular disease viruses. It is still recommended that the animal disease tests be carried out in the field where suspicious cases are encountered, samples being sent to the laboratory for confirmation and typing should this be necessary.

*Piroplasmosis (Texas fever) of cattle.*—Piroplasmosis is a common disease of cattle in many parts of the world. The cause is a sporozoan parasite of red blood cells. It is transmitted by arthropods (arachnids) commonly called ticks. The disease is known by many names: bovine malaria, tick fever, southern cattle fever, babesiosis, piroplasmosis, and Texas fever, the latter name being best known in the United States. This name was attached to the disease in 1868 when a large shipment of Texas range cattle was made up the Mississippi River by boat to Cairo, Illinois, from whence they were distributed widely through the states of Illinois and Indiana (6). Wherever these cattle went the native animals with which they had contact sickened and died, whereas Texas cattle seemed perfectly well. Enormous losses were incurred. The cause of the malady was not known or even suspected at the time, but the fact seemed to be clear that the disease came with the Texas cattle. Other experiences made it clear that cattle from the southern part of the country could not be mixed with impunity with animals originating from the north, since the latter would sicken and die. Beginning in 1883, Salmon began drawing the "Texas Fever Line," this being a line across the map of the United States which roughly approxi-

mated the Mason and Dixon line of earlier days and divided the uninfected territories of the north from the infected of the south. The line really marked the limit of distribution of the so-called Texas fever tick, but that fact was unrecognized at the time. Some of the livestock men believed that the common southern cattle tick (*Margaropus annulatus*) played a role in Texas fever, but several commissions that studied the situation ridiculed the idea. Smith & Kilborne (7) eventually showed that the cause of the disease could be found in the blood corpuscles, and Curtice (8) demonstrated that the southern cattlemen had been right in their suspicion of the tick. It was shown that the disease had no way of passing from one host to another except through the intermediary host, the fever tick. This discovery made it clear that if a means of eliminating this tick could be found, the disease could be eradicated.

By 1906, methods of destroying the cattle tick had been developed and a nationwide program for eliminating Texas fever was inaugurated. Beginning for the most part near the Texas fever line and working southward, all cattle and other animals on which the fever tick subsisted were systematically dipped in chemical solution which destroyed the ticks. Each animal had to be dipped on several occasions to destroy new crops of ticks which attached themselves from the vegetation of the infected pasture lands when the animals were turned back to graze. Eventually all ticks were destroyed, and since the disease could not spread in the absence of its only transmitting agency, it died out. The early chemical dips were not as effective as the arsenical dips that were introduced in 1911. These were used throughout the remainder of the campaign.

The Texas fever eradication program proceeded steadily but rather slowly at times, since many state laws were inadequate for the job to be done, state officials were not always cooperative, funds frequently were short and, in some areas, there was active and often violent opposition to the work. Many dipping vats were destroyed by dynamite, cattle often had to be dipped while recalcitrant owners were restrained by law enforcement officers, and a few workers were killed by irate citizens. Eventually the public was won over to the importance of the work. Within two decades, more than two-thirds of the territory which originally had been infected was released from quarantine. By 1940, the disease had been wholly eliminated from the United States except for a small strip of territory along the Rio Grande River and westward which occasionally harbors infection in undipped cattle illegally moved across the border from Mexico.

The elimination of Texas fever has been a great boon to the South. Formerly it was impractical to take fine breeding stock from uninfected regions into the southern states to improve their cattle, for most of the imported stock promptly died of Texas fever. Now that this deterrent is no longer present, the herds of the South have been greatly improved and the region has become a great livestock producing area.

*Dourine.*—Dourine is a chronic destructive disease of horses caused by *Trypanosoma equiperdum.* Unlike most trypanosome diseases which are transmitted by blood-sucking arthropods, dourine is a true venereal disease, since it is transmitted almost wholly to mare from stallion, and vice-versa, by coitus.

Dourine formerly was common in Europe, especially in the more southerly countries. It was first recognized in the United States in 1885 in the state of Illinois to which it had been introduced in a stallion imported from France (9). The disease was later found in many of the western states. By 1903 the disease had become so widespread that it was decided to organize an eradication campaign. This involved the tracing of all cases of the disease, the slaughter of all infected females, and the castration or slaughter of all infected males. At first the work made slow headway, but when the complement-fixation test was introduced about 1912 as a means of diagnosis, the disease was rapidly brought under control. By 1920, the disease was thought to have been eradicated; however, in 1941 a small focus of infection was found in Arizona in horses on an Indian reservation. These animals have been eliminated and it is believed that the country is now free from this disease.

*Bovine tuberculosis.*—Tuberculosis has long been prevalent in Europe, especially in the southern half of that continent and in the British Isles. From these regions, from which all of the original breeding stock of our principal dairy and some of our beef cattle breed came, the disease was imported into this country. It can hardly be doubted that cases were imported in the very earliest cattle that arrived, but there is little information on this score. In any case it is definitely known that many tuberculous cattle were imported in the period after our Civil War and before 1900, when the tuberculin test began to be used as a means of excluding them. At the end of the nineteenth century the disease was very common in this country and increasing rapidly, particularly in dairy cattle, and especially in the eastern states where most of the imported cattle first found homes. From the eastern states the disease spread westward. Beef cattle never were extensively infected, and the disease did not spread widely among these animals because of the free open life that they led. Tuberculosis among animals, as well as man, is a disease of overcrowding and poor housing.

The tuberculin test was first used on animals in this country by a Commission from the University of Pennsylvania (10) in 1891. Within a few years it was used quite extensively, through sporadically, by owners who wished to free their herds of the disease. Application of the tuberculin test on a large scale was not undertaken until the Federal-State Cooperative Plan for the Eradication of Bovine Tuberculosis came into existence in 1917. This plan envisaged the systematic tuberculin testing of all dairy animals, and sample testing of beef cattle herds, with the removal and slaughter of all reacting animals, the disinfection of all premises upon which affected

cattle had lived, and the payment of indemnity by the State and Federal governments to enable owners to replace their infected animals with disease-free stock.

The plan developed rather slowly during the first five years, then accelerated rapidly. In 1924, it was estimated that the average incidence of the disease was about 4 per cent but in some states it had risen to as high as 25 per cent. In 1940, all states had joined the plan, and the average incidence of the disease had been reduced to an estimated 0.46 per cent, a reduction of about 85 per cent. Additional progress has been made since, but the disease has not been eliminated. As an economic and public health hazard, bovine tuberculosis is no longer important in this country. This campaign has cost more money and resulted in the slaughter of more animals than any other animal-disease control program in history. There can be no question, however, that the results have justified its cost. Canada, the British Isles, and the Scandinavian countries have carried on similar programs and the results have been equally encouraging.

*Bovine brucellosis.*—Contagious abortion, Bang's disease, or brucellosis of cattle was identified as a specific infectious disease in Europe in 1897 (11). It was found in this country shortly afterwards. It is clear that it had existed long before its nature was recognized.

This disease does not kill cattle but it causes abortions, loss of calves, and diminution of the milk yield. Infected cattle and their milk present a hazard to man since *Brucella abortus,* the cause of bovine brucellosis, is also one of the causes of the disease of man known as undulant fever.

It was recognized that this disease was spreading and becoming a serious hazard in dairy herds prior to the first World War. In 1926, it became known that the disease also affected man through contact and by drinking raw milk (12). At about this time, too, the agglutination test which had been used for a decade or more became generally recognized as an accurate means of detecting animals that harbored the disease. Many of the states then began to require that cattle imported from abroad and from other states be accompanied by certificates indicating that they had recently been tested by the agglutination test and found negative to it. This forced breeders of purebred stock, who often wished to sell animals outside their own state, to have their herds tested and to eliminate the reacting animals. Since most states had no regulations with reference to moving animals from farm to farm within their own borders, reacting animals thus were sold to any who would buy and who lived in the same state. This resulted in scattering the disease to many herds that previously had not been infected. Many of the purebred herds were freed of brucellosis prior to 1940 as a result of the use of the agglutination test and the elimination of reactors, but as long as owners of such herds had neighbors with cattle that were not free from this disease, it proved to be difficult to maintain uninfected herds for very long, especially if they contained many animals.

In 1930, Buck (13) introduced a method of dealing with brucellosis which has proved to be very useful. A culture of *Brucella abortus*, which originally had been of normal virulence, was found to have lost most of its virulence for cattle. Since it appeared to have retained excellent antigenicity it was tried as a vaccine strain. This culture was identified as Strain 19. Injected intravenously into pregnant cattle in very large doses, Strain 19 will usually cause abortions. In smaller doses, injected subcutaneously, it does no harm but confers strong protection against exposure to virulent infection. When used on adult cattle, a strongly positive agglutination test will be engendered and this reaction generally will persist for several years and even longer. If it is used on young calves from four to eight months of age, a good measure of resistance will be carried over into the period of young adulthood, but the agglutination test which is highly positive soon after the vaccine is given gradually fades. In most animals the positive titer will have disappeared by the time the animal has reached breeding age.

Calfhood vaccination is now extensively used in the United States and elsewhere. Strain 19 is by far the most satisfactory strain that has been found for the purpose. It is no longer used extensively in adult cattle, since equally good results seem to be secured by using it on calves between four and eight months of age, and there are few persisting blood-agglutination titers in such animals to cause confusion with later blood tests.

At the present time there is a nationwide campaign, again a State-Federal plan, to eliminate brucellosis from our cattle. This plan has made rapid progress. As of this writing (December, 1957) nine states are certified to be brucellosis-free, and seven others expect to reach this goal by July 1, 1958. The majority of the states have target dates on or before January 1, 1960.

The plan is based upon systematic vaccination of all calves to reduce susceptibility to the disease and thus discourage its spread, the gradual surveying of all herds by the agglutination test, and the removal and slaughter of all reacting animals. The surveying of dairy herds for the presence of the disease has been greatly hastened and cheapened by the introduction of what is known as the milk-ring-test (MRT). This test can be done on the pooled milk of entire herds and it serves to indicate the herds which harbor the infection and those which are free from it. It eliminates the necessity of testing individual animals on the original survey. Individual blood tests must then be done only on animals in herds in which infection is indicated by the milk-ring-test.

The concerted attack on this disease has already reduced its incidence by more than 75 per cent. The general practice of calfhood vaccination has very nearly eliminated the economic losses, and human infections are much fewer today than they were only a decade ago. We can confidently look forward to the near elimination of bovine brucellosis in this country by 1960 and perhaps eradication within another decade.

*Vesicular exanthema of swine.*—In 1932, an outbreak of what was thought to be foot-and-mouth disease appeared in Southern California. The disease occurred only in swine. It was handled as previous outbreaks of foot-and-mouth disease had been, that is, by the radical method of slaughter and burial. While this was going on, tests of the virus disclosed that it was infective only for swine; that cattle horses, and guinea pigs were resistant. First regarded as an aberrant strain of foot-and-mouth disease virus, one which had lost virulence for all species except the one in which it occurred, it finally was decided by Traum (14) that the virus was different and that the original diagnosis of foot-and-mouth disease had been an error. Traum named this disease vesicular exanthema.

In the course of time it became apparent that the disease was enzootic in California. For 20 years after the original outbreak, it appeared sporadically in swine herds in the southern half of the state. Only once during this period was there a time lapse greater than one year when no cases were reported. It was recognized very early that the disease was found primarily in pigs that were fed on garbage, and that grain-fed pigs were very seldom involved. It became clear that the virus occurred in garbage in fragments of uncooked pork, and this meant, of course, that pigs having the disease were being slaughtered for market. The slaughter-house inspectors watched for evidence of this disease in animals bought for slaughter and refused to allow them to be brought into the establishment when the malady was recognized but, of course, there were pigs in the very early stages of the disease that contained virus but did not exhibit recognizable symptoms or lesions.

Until 1952, vesicular exanthema did not appear elsewhere than in California, because the people of this state consumed more pork than they produced and no infected materials were shipped out. In 1952, however, 20 years after the disease was first recognized, vesicular exanthema suddenly appeared in the State of Nebraska. It was traced to a garbage-feeding farm near Laramie, Wyoming, which obtained part of its garbage from the dining cars of transcontinental trains. It is presumed that the disease was introduced in materials originating in California. Before the disease had been recognized in the Nebraska animals, it had been spread through the shipping of infected animals to many places. Within a few weeks the disease had been diagnosed in 42 states and the District of Columbia.

Vesicular exanthema causes substantial losses, in morbidity rather than mortality. More feared than the disease itself, however, was the possibility that outbreaks might mask foot-and-mouth disease. It was this fact that stimulated both State and Federal governments to institute measures for eradication of the disease. Since it is less contagious than foot-and-mouth disease, it was not necessary to use measures as drastic as those used for the latter. Infected premises were quarantined, sick animals destroyed, and

no unaffected stock was permitted to be moved, other than to processing establishments where the meat was cooked before entering the trade. In many cases animals were retained on the original premises until all evidence of infection had disappeared and it was reasonably certain that the virus was eliminated. Also, all states, except one, enacted laws which required commercial garbage-feeding establishments to cook all garbage before it was fed. The U. S. Public Health Service also issued regulations which denied access to interstate trade of all pork from swine which at any time in their lives had been fed raw garbage. These measures have apparently been successful in eliminating vesicular exanthema, since no cases have been recognized in more than a year. The same measures also should greatly reduce the incidence of trichinosis and cholera in swine, since garbage feeding has been an important influence in propagating both of these diseases.

*Scrapie in sheep and goats.*—Scrapie is a chronic, fatal disease of the nervous system. So far as is known, it affects only sheep and goats. For more than 200 years it has prevailed in the British Isles, and it was known on the European mainland even earlier. The disease spreads slowly but when it becomes well established the losses are serious. The causative factor is unknown. Some have thought that genetic factors were involved since certain breeds are affected more often than others. It is not certain, however, that this difference may not be merely a manifestation of the degree of exposure. More recent work has pointed to the existence of a virus as the etiologic agent, and this concept is generally held today. Apparently it has been transmitted somewhat erratically by inoculation. Certainly, if scrapie is a virus-induced disease, the causative agent is a very unusual one.

Scrapie was unknown in the United States until 1947 when a case was recognized in the State of Michigan (15). In 1952, cases were recognized in California (16) and within the next few months others were found in Ohio, Illinois, New York, and Connecticut. Nearly all cases have been in purebred Suffolk sheep, and practically all have been traced to a few Canadian flocks which contained stock imported from the British Isles.

An effort is now being made to eradicate this disease. Importation of exposed animals is prohibited, all known exposed flocks in the United States have been quarantined, all affected animals are destroyed as soon as they are recognized, and a great many flocks containing possible contacts but having no recognized cases in recent years are under continuing surveillance. Since there are no means of diagnosing this disease except through clinical symptoms, and since the incubation period is very long and variable, affected animals may have lived in several different flocks, perhaps leaving infection in each, before they are recognized to be suffering from scrapie. The success of the efforts to eliminate scrapie from this country is presently in doubt. The results appear to be encouraging but an accurate appraisal of the situation cannot be made at this time.

*Rabies.*—Efforts to control rabies in the United States have not met with great success except in local areas. There is evidence that the disease existed in Colonial days in Massachusetts and other colonies. Whether it had been imported from Europe in dogs, or whether it existed in wildlife before the arrival of the early European settlers is not known. At the present time rabies occurs in all parts of the country except in the New England States, and in one or two states of the northwestern part of the country. The disease occurs in all warmblooded animals, but in general it is transmitted only by biting species, since the saliva is the only means by which the virus escapes from the body. Horses, cattle, swine, sheep, and wild herbivora often become infected and die with rabies, but such species seldom transmit the disease to others. Most human cases are caused by bites of rabid dogs and cats; occasionally wolves and other wild carnivora are involved.

In the United States, the principal reservoirs of rabies virus are in wild animals. In many areas it is in foxes; in others it is in skunks, coyotes, and squirrels. The significance of the finding of rabies in free-living insectivorous bats in at least 15 widely scattered states is still to be evaluated (17). Antirabic vaccination of dogs has greatly reduced the incidence of canine rabies in many parts of the country, and this has also served to reduce the incidence of human rabies. Efforts of disease-control and conservation officials to eliminate rabies from wild life have met with only limited success. The prospect for the eradication of this disease in the near future is not encouraging.

*Hog cholera.*—Cholera has been the most destructive disease of swine in the United States for more than a century. Since 1908, when Dorset, Niles & McBryde (18) introduced an immunization method involving the use of active virus and antiserum simultaneously, the disease may be said to have been brought under control, which, however, has been expensive and somewhat uncertain. No progress toward eradication has been achieved.

There are some who believe that hog cholera could be eliminated if the field use of active virus was banned, and if outbreaks were handled in the way that those of foot-and-mouth disease are handled in this country, that is, by slaughter and burial of all infected herds and the payment of indemnity to owners for their losses. These methods have been used successfully in Canada for many years, and also in some European countries where the disease is sporadic. There are other equally competent authorities who do not agree that the disease could be eradicated in this way under the conditions that prevail in the United States. Small experiments are now being conducted in some southern states to test the idea. We shall have to wait for several years before their success may be judged.

### Infectious Disease Problems Remaining to be Solved

In concluding this paper on the efforts that have been made to control and eradicate infectious diseases of animals in the United States, I should like to make it clear that while we have come a long way, the task is far from finished. There are some important diseases of livestock, such as bovine mastitis, that are now being controlled, at least partially, but are of such a nature that there is no hope of ever eradicating them. Others, such as swine influenza, probably could be controlled but the necessary procedures are not economically feasible. There are others, such as atrophic rhinitis of swine, for which we have no methods of control. For some diseases such as anthrax and blackleg, we have not hopes of eradication but we have satisfactory methods of prophylactic immunization. For others, such as coccidiosis in the various species, blackhead in turkeys, and many bacterial infections that formerly exacted heavy losses, antibiotics and the sulfa drugs have performed the same kind of miracles that are well-known in many human diseases. We are developing better methods of preventing distemper in dogs. Brucellosis in swine, which is caused by a different organism than the one which causes the disease in cattle, is proving a rather stubborn malady to combat, yet progress is being made. Eradication cannot be hoped for in the near future, however, unless new approaches are found. We still have a host of diseases that produce significant losses in cattle, swine, and poultry, yet it is indicative of progress that the diseases which are the cause of principal concern today are, in many instances, ones that we did not even recognize 25 years ago, and the ones that were of concern then are in nearly every instance under good control today. At the present time it can safely be said that no area of the earth is safer than the United States, as far as infectious disease losses are concerned, for the growing of livestock.

### LITERATURE CITED

1. Simms, B. T., *Animal Diseases, Yearbook Agr.* (*U.S. Dept. Agr.*), p. 54 (1956)
2. Henning, M. W., *Animal Diseases in South Africa,* 2nd ed., 171 (Central News Agency, Ltd., Pretoria, South Africa, 879 pp., 1949)
3. Houck, U. G., *Bureau of Animal Industry, U.S. Dept. Agr.,* p. 41 (Published by author, Washington, D.C., 390 pp., 1924)
4. Mohler, J. R., and Eichhorn, A., *U.S. Dept. Agr., Bull. No. 136* (Government Printing Office, Washington, D.C., 1911)
5. Brooksby, J. B., *Agriculture* (*London*), *Rept. Ser., No. 12* (H. M. Stationery Office, London, 40 pp., 1952)
6. Moore, V. A., *Pathology and Differential Diagnosis of the Infectious Diseases of Animals,* 4th ed., 327 (The Macmillan Co., New York, 593 pp., 1916)
7. Smith, T., and Kilborne, F. L., *Bureau of Animal Industry, U.S. Dept. Agr., Bull. No. 1* (Government Printing Office, Washington, D.C., 1893)

8. Curtice, C., *J. Comp. Med. Vet. Arch.*, **13**, 1 (1892)
9. Williams, W. L., *Am. Vet. Rev.*, **12**, 295 (1888)
10. Anonymous, *Am. Vet. Rev.*, **15**, 431 (1891)
11. Bang, B., *J. Comp. Pathol. Therap.*, **10**, 125 (1897)
12. Carpenter, C. M, and Merriam, H. E., *J. Am. Med. Assoc.*, **87**, 1269 (1926)
13. Buck, J. M., *J. Agr. Research*, **41**, 667 (1930)
14. Traum, J., *J. Am. Vet. Med. Assoc.*, **88**, 316 (1936)
15. Thorp, F., Judd, A. W., Gray, M. L., and Sholl, L. B., *Mich. State Coll. Veterinarian*, **13**, 36 (1952)
16. Boyd, A. G., *Proc. Ann. Meet. U.S. Livestock Sanitary Assoc.*, 56th Meeting, 254 (1952)
17. Enright, J. B., *Ann. Rev. Microbiol.*, **10**, 369 (1956)
18. Dorset, M., McBryde, C. N., and Niles, W. B., *Bureau of Animal Industry, U.S. Dept. Agr., Bull. No. 102* (Government Printing Office, Washington, D.C., 1908)

# METABOLISM OF CARBOHYDRATES AND RELATED COMPOUNDS[1,2]

By S. R. Elsden and J. L. Peel
*Agricultural Research Council Unit for Microbiology*
*Microbiology Department, University of Sheffield,*
*Sheffield, England*

Until about 1950, the Embden-Meyerhof mechanism for the breakdown of hexoses appeared to be the only one of any significance. Since then, a number of new pathways have been discovered and shown to be exceedingly important. This breakthrough, resulting primarily from the work of Cohen, Dickens, Doudoroff, Horecker, Racker, and their colleagues, led to a great outburst of activity. The various enzymes concerned were sought and found in a wide variety of organisms and previous reviewers were confronted with the enormous task of presenting the "news," much of it "stop-press," in a readable form. One has only to peruse their articles (1 to 4) to see how well they have succeeded. We have formed the impression that activity is now diminishing in this area and we have come to the conclusion that it would not be inappropriate to attempt to put the many findings into perspective and to consider the physiological significance of these new pathways of carbohydrate catabolism.

Over the past few years, there has been a notable increase in the number of reports on polysaccharide synthesis and, accordingly, some emphasis is placed on this topic. There is also a renewed interest in the metabolism of 2-carbon acids and, while this topic is to be reviewed in the 1959 volume by H. L. Kornberg, the recent findings are of such importance as to demand immediate consideration. This is, in fact, our "stop-press" item. Also included is a section on the metabolism of aromatic compounds. Although this topic has been reviewed recently by Evans (5), it has not been dealt with in this series for some time and a considerable number of interesting papers have appeared in the past year. Finally, under the heading of "Miscellaneous" certain aspects of metabolism which do not fit conveniently into any of the above sections are discussed.

Reviews on the following topics have appeared during the year but are

[1] The survey of the literature pertaining to this review was concluded in December, 1957.

[2] The following abbreviations will be used: ADP (adenosine diphosphate); AMP (adenosine monophosphate); ATP (adenosine triphosphate); CoA (coenzyme A); DNP (2,4-dinitrophenol); DPN (oxidized diphosphopyridine nucleotide); DPNH (reduced disphosphopyridine nucleotide); GSH (reduced glutathione); $P_i$ (inorganic phosphate); UDP (uridine diphosphate); UMP (uridine monophosphate); UTP (uridine triphosphate).

not cited in the text: β-galactosidase (6); the thiobacilli (7); the biochemistry of spores (8); and carbon dioxide fixation (9). In addition, Barker (10) has published in book form a series of lectures covering his work on fermentation.

It has become almost traditional for reviewers to bewail the ever increasing output of work; we, however, prefer to rejoice, for this may be taken as evidence of an increasing interest in microorganisms. In common with some previous reviewers, no attempt is made in this review to effect a comprehensive survey of the year's publications; we have restricted it to selected topics. The choice of material has been directed on the one hand by the writers' particular interests, and on the other by the availability of the journals. In addition, reports have been eschewed of the demonstration in yet another organism, of enzymes already known to be widely distributed, except where such observations are of importance in relation to new concepts. Abstracts of oral communications which usually give conclusions rather than data have generally been ignored. For deliberate omissions no apology is offered but to those whose work has, by accident, been overlooked, the words of Quince from *A Midsummer Night's Dream* (11) are humbly offered:

> If we offend, it is with our goodwill.
> That you should think we come not to offend
> But with goodwill. To show our simple skill,
> That is the true beginning of our end.

## Energetics of Carbohydrate Metabolism

Our knowledge of the mechanisms concerned with the anaerobic breakdown of carbohydrates and related compounds is now reaching the point where it is possible to draw up equations which not only depict the reactions involved but which also indicate precisely the amount of energy made available in terms of moles ATP/mole of substrate used. Unfortunately, the same cannot be said of aerobic organisms in which phosphorylation is known to be coupled to electron transport, for in this case neither the mechanisms involved nor the actual yields of ATP are known. Since ATP is now generally believed to be the major energy mediator in the cell, a discussion of the anaerobic breakdown of carbohydrates from the point of view of ATP yields appears pertinent.

The oldest established mechanism is that occurring in yeasts and the homofermentative lactic acid bacteria, both of which convert hexoses to pyruvate via fructose-1-6-DP. The characteristic feature of this pathway is the cleavage of fructose-1-6-DP by aldolase and in these organisms the subsequent reactions of pyruvate are not associated with ATP synthesis. The sole source of ATP therefore, when these organisms are grown anaerobically upon hexoses, is the Embden-Meyerhof pathway which yields 2 moles ATP/mole hexose fermented (Equations 1a to 1e).

$$\text{Hexose} + 2\,\text{ATP} \longrightarrow \text{Fructose-1-6-DP} + 2\,\text{ADP} \quad \text{1a.}$$
$$\text{Fructose-1-6-DP} \longrightarrow 2\,\text{Triose-P} \quad \text{1b.}$$
$$2\,\text{Triose-P} + 2\,P_i + 4\,\text{ADP} \rightarrow 2\,\text{Pyruvate} + 4(\text{H}) + 4\,\text{ATP} \quad \text{1c.}$$
$$2\,\text{Pyruvate} + 4(\text{H}) \longrightarrow 2\,\text{Lactate} \quad \text{1d.}$$
$$\text{or}$$
$$2\,\text{Ethanol} + 2\,CO_2$$

$$\text{Hexose} + 2\,P_i + 2\,\text{ADP} \longrightarrow 2\,\text{Lactate} + 2\,\text{ATP} \quad \text{1e.}$$
$$\text{or}$$
$$2\,\text{Ethanol} + 2\,CO_2 + 2\,\text{ATP}$$

A second mechanism of hexose breakdown, commonly referred to as the Entner-Doudoroff pathway, was first discovered in the aerobic *Pseudomonas saccharophila* (12, 13), and it was only later suggested that it operates anaerobically in *Pseudomonas lindneri,* which ferments glucose to ethanol and carbon dioxide. Hexose is converted via glucose-6-P to 6-phosphogluconate which is then dehydrated to give 2-keto-3-deoxy-6-phosphogluconate, the substrate of the cleavage enzyme. The products formed by the cleavage enzyme are pyruvate and triose-P and the overall reactions are given in Equations 2a to 2g.

$$\text{Hexose} + \text{ATP} \longrightarrow \text{Glucose-6-P} + \text{ADP} \quad \text{2a.}$$
$$\text{Glucose-6-P} \longrightarrow \text{6-phosphogluconate} + 2(\text{H}) \quad \text{2b.}$$
$$\text{6-Phosphogluconate} \longrightarrow \text{2-Keto-3-deoxy-6-phosphogluconate} + H_2O \quad \text{2c.}$$
$$\text{2-Keto-3-deoxy-6-phosphogluconate} \rightarrow \text{Pyruvate} + \text{Triose-P} \quad \text{2d.}$$
$$\text{Triose-P} + P_i + 2\,\text{ADP} \longrightarrow \text{Pyruvate} + 2\,\text{ATP} + 2(\text{H}) \quad \text{2e.}$$
$$2\,\text{Pyruvate} + 4(\text{H}) \longrightarrow 2\,\text{Ethanol} + 2\,CO_2 \quad \text{2f.}$$

$$\text{Hexose} + \text{ADP} + P_i \longrightarrow 2\,\text{Ethanol} + 2\,CO_2 + \text{ATP} \quad \text{2g.}$$

This reaction sequence yields 1 mole ATP/mole hexose and is thus only half as efficient, in terms of ATP production, as the Embden-Meyerhof system. Gibbs & DeMoss (14) studied the distribution of $^{14}C$ in the ethanol and carbon dioxide when specifically labelled glucoses were fermented by *Pseudomonas lindneri.* The distribution was that predicted by the Entner-Doudoroff pathway. More recently, Sokatch & Gunsalus (15) have studied the fermentation of gluconate by *Streptococcus faecalis* in which gluconate is converted to lactate and carbon dioxide according to Reaction 3.

$$6\,\text{Gluconate} \rightarrow 11\,\text{Lactate} + 3\,CO_2 \quad \text{3.}$$

The fermentation of gluconate is adaptive and they found that the aldolase which cleaves 2-keto-3-deoxy-6-phosphogluconate is only present in organisms which have been grown on gluconate [see also Sokatch, Prieto & Gunsalus (16)]. On the basis of this and other evidence which will be considered later they concluded that part of the gluconate is degraded via the Entner-Doudoroff pathway.

Recent studies of the fermentation of pentoses have revealed a third cleavage mechanism. *Lactobacillus pentoaceticus* and *Lactobacillus pentosus*

ferment pentoses with the formation of one mole each of acetate and lactate/mole of pentose fermented. Tracer experiments showed that $C_1$ and $C_2$ of acetate are derived from $C_2$ and $C_1$, respectively, of the pentoses and that $C_1$, $C_2$, and $C_3$ of the lactate come from $C_3$, $C_4$, and $C_5$ of the pentose (17, 18). This fermentation of pentose thus involves a cleavage between $C_2$ and $C_3$. There is good evidence to support the view that the fermentation of glucose by *Leuconostoc mesenteroides* involves the conversion of glucose to a pentose phosphate followed by cleavage of the pentose between $C_2$ and $C_3$, yielding ethanol and lactate instead of the acetate and lactate produced from pentose (19, 20). An extract has been prepared from pentose-grown *L. pentosus* by Heath, Hurwitz & Horecker (21), which cleaves xylulose-5-P to acetyl-P and triose-P. This new cleavage enzyme, which has been called phosphoketolase (22), requires inorganic-P, diphosphothiamine, $Mg^{++}$, and a thiol compound for its activity (mercaptoethanol, GSH, cysteine, and thioglycollate have been found effective). The reaction, which is not reversible, is represented by Equation 4b. Phosphate could be replaced by arsenate, in which case acetate was formed instead of acetyl-P. It would seem reasonable to suppose that, initially, the substrate is converted to triose-phosphate and a bound form of glycolaldehyde. The glycolaldehyde-enzyme complex is converted to an acetyl-enzyme one and the acetyl group so formed is transferred to a suitable acceptor. The first step in the reaction thus resembles that catalyzed by transketolase which also requires both $Mg^{++}$ and diphosphothiamine. The need for a thiol compound suggests that sulphydryl groups on the enzyme play a part; hence there is an obvious parallel between this enzyme and triosephosphate dehydrogenase. Purified preparations were specific for xylulose-5-P and neither fructose-6-P nor sedoheptulose-7-P were attacked. On the other hand, Schramm & Racker (23) have reported that extracts of *Acetobacter xylinum* contain a similar enzyme which cleaves fructose-6-P giving acetyl-P and erythrose-4-P. The action of phosphoketolase is very complex and it will be interesting to see whether a single enzyme or a multienzyme system is involved. The fact that highly purified preparations have been made (22) suggests that the activity is associated with a single protein.

In the presence of ADP, crude extracts formed acetate and ATP from xylulose-5-P. The extracts contained acetokinase which, like phosphoketolase, was only formed when the organisms were grown on pentoses (xylose and arabinose were used). Heath *et al.* (21) point out that in the fermentation of pentoses by this mechanism there is likely to be a net gain of 2 moles ATP/mole pentose fermented. This is made clear by Equations 4a to 4f.

$$\text{Pentose} + \text{ATP} \longrightarrow \text{Xylulose-5-P} + \text{ADP} \qquad 4a.$$
$$\text{Xylulose-5-P} + P_i \longrightarrow \text{Triose-P} + \text{Acetyl-P} \qquad 4b.$$
$$\text{Acetyl-P} + \text{ADP} \longrightarrow \text{Acetate} + \text{ATP} \qquad 4c.$$
$$\text{Triose-P} + P_i + 2\,\text{ADP} \rightarrow \text{Pyruvate} + 2(\text{H}) + 2\,\text{ATP} \qquad 4d.$$
$$\text{Pyruvate} + 2(\text{H}) \longrightarrow \text{Lactate} \qquad 4e.$$

$$\text{Pentose} + 2\,P_i + 2\,\text{ADP} \rightarrow \text{Lactate} + \text{Acetate} + 2\,\text{ATP} \qquad 4f.$$

If, as would seem probable, the *Leuconostoc* fermentation of glucose proceeds along similar lines and the tracer evidence indicates that this is so, then the reaction sequence will be:

$$\text{Glucose} + \text{ATP} \longrightarrow \text{Glucose-6-P} + \text{ADP} \quad 5a.$$
$$\text{Glucose-6-P} \longrightarrow \text{Xylulose-5-P} + CO_2 + 4(H) \quad 5b.$$
$$\text{Xylulose-5-P} + P_i \longrightarrow \text{Acetyl-P} + \text{Triose-P} \quad 5c.$$
$$\text{Acetyl-P} + 4(H) \longrightarrow \text{Ethanol} + P_i \quad 5d.$$
$$\text{Triose-P} + P_i + 2\,\text{ADP} \rightarrow \text{Pyruvate} + 2(H) + 2\,\text{ATP} \quad 5e.$$
$$\text{Pyruvate} + 2(H) \longrightarrow \text{Lactate} \quad 5f.$$

$$\text{Glucose} + P_i + \text{ADP} \longrightarrow \text{Ethanol} + \text{Lactate} + \text{ATP} + CO_2 \quad 5g.$$

It will be seen that when glucose is fermented by *L. mesenteroides* there is a net gain of 1 ATP/mole glucose fermented in contrast to a net gain of 2 moles ATP when pentose is the substrate. The loss of ATP occurs as a result of the reduction of acetyl-P to ethanol. Dawes & Foster (24) have shown that in *Escherichia coli* acetyl-CoA rather than acetyl-P is the substrate of the reducing enzyme [cf. Burton & Stadtman (25)].

The enzymic basis of the separation of the lactobacilli into homofermenters and heterofermenters is now clear. In the former, the glucose is fermented via the Embden-Meyerhof route with aldolase as the cleavage enzyme; in the latter, glucose is converted to xylulose-5-P which is then cleaved by phosphoketolase. Buyze, van den Hamer & de Haan (26) have studied the distribution of aldolase, glucose-6-P dehydrogenase, and 6-phosphogluconate dehydrogenase in lactobacilli. They found that all homofermentative species contained aldolase, an enzyme characteristic of the Embden-Meyerhof pathway, whereas no heterofermentative species contained this enzyme. All heterofermentative species contained both glucose-6-P dehydrogenase and 6-phosphogluconate dehydrogenase and these two enzymes were also present in some of the homofermenters. It is hoped that these authors will go on to examine the distribution of both phosphoketolase and phosphofructokinase in lactobacilli; the distribution of the latter enzyme is of interest since, like aldolase, it is characteristic of the Embden-Meyerhof pathway.

A further mechanism of fermentation of pentoses involves a conversion of the pentoses, via the 5-phosphate to fructose-6-P and triose-P by transketolase and transaldolase, and the fructose-6-P so produced is broken down by the Embden-Meyerhof mechanism. Evidence has been obtained that pentoses are fermented in this way by *E. coli* (27), by *Aerobacter cloacae* (28, 29, 30) and by clostridia (31).

The pentose cycle is also involved in the fermentation of gluconate by *Streptococcus faecalis*. This fermentation which has already been mentioned briefly (p. 147), will now be discussed in more detail. The amounts of lactate and carbon dioxide produced did not quite fit Equation 3, but when the fermentation was carried out in the presence of $10^{-3}M$-arsenite, a more precise agreement was observed. Fermentation of gluconate-1-$^{14}$C or gluconate-2-$^{14}$C showed that all the carbon dioxide was derived from gluconate-$C_1$.

Gluconate-2-$^{14}$C gave lactate labelled on all three positions with $C_2$ containing 40 to 60 per cent of the isotope, depending upon conditions. Taking into consideration the stoichiometry of the fermentation, the adaptive formation of the aldolase which cleaves 2-keto-3-deoxy-6-phosphogluconate, and the data from the isotope experiments, Sokatch & Gunsalus (15) have suggested the following scheme: Gluconate is first phosphorylated to give 6-phosphogluconate; half of this is oxidized to a pentose-5-P (ribulose-5-P?) which is then converted to fructose-6-P and triose-P by transketolase and transaldolase. The fructose-6-P so formed is then converted to fructose-1-6-DP which is broken down via the Embden-Meyerhof pathway. The other half of the 6-phosphogluconate is dehydrated to 2-keto-3-deoxy-6-phosphogluconate which is then cleaved to pyruvate and triose-P. This scheme is summarized by Equation 6a to 6i.

$$6\ \text{Gluconate} + 6\ \text{ATP} \longrightarrow 6(\text{6-Phosphogluconate}) + 6\ \text{ADP} \quad 6a.$$
$$3\ \text{Phosphogluconate} \longrightarrow 2\ \text{Fructose-6-P} + \text{Triose-P} + 3\ CO_2 + 6(H) \quad 6b.$$
$$2\ \text{Fructose-6-P} + 2\ \text{ATP} \longrightarrow 2\ \text{Fructose-1-6-DP} + 2\ \text{ADP} \quad 6c.$$
$$2\ \text{Fructose-1-6-DP} \longrightarrow 4\ \text{Triose-P} \quad 6d.$$
$$3\ \text{Phosphogluconate} \longrightarrow 3(\text{2-Keto-3-deoxy-6-phosphogluconate}) + 3\ H_2O \quad 6e.$$
$$3(\text{2-Keto-3-deoxy-6-phosphogluconate}) \rightarrow 3\ \text{Triose-P} + 3\ \text{Pyruvate} \quad 6f.$$
$$8\ \text{Triose-P} + 8\ P_i + 16\ \text{ADP} \longrightarrow 8\ \text{Pyruvate} + 16\ \text{ATP} + 16(H) \quad 6g.$$
$$11\ \text{Pyruvate} + 22(H) \longrightarrow 11\ \text{Lactate} \quad 6h.$$

$$6\ \text{Gluconate} + 8\ P_i + 8\ \text{ADP} \longrightarrow 11\ \text{Lactate} + 3\ CO_2 + 8\ \text{ATP} \quad 6i.$$

Thus, gluconate fermentation by *S. faecalis* yields 1.33 ATP/mole gluconate fermented. It will be seen that, when pentose is fermented via hexose and the Embden-Meyerhof route the net gain of ATP up to the pyruvate stage will be 1.67 moles ATP/mole pentose fermented.

$$3\ \text{Pentose} + 5\ P_i + 5\ \text{ADP} \rightarrow 5\ \text{Pyruvate} + 10(H) + 5\ \text{ATP} \quad 7.$$

The net gains of ATP when monosaccharides are fermented by the various pathways outlined above are given in Table I. It must be stressed that these yields apply only to those organisms which metabolize pyruvate without the formation of high-energy acetyl compounds, i.e., acetyl-P and

TABLE I

ATP YIELDS FROM THE ANAEROBIC BREAKDOWN OF MONOSACCHARIDES

| Substrate | Pathway | ATP yield (mole/mole substrate) |
|---|---|---|
| Hexose | Embden-Meyerhof | 2 |
| Hexose | Entner-Doudoroff | 1 |
| Hexose | Phospho-Ketolase | 1 |
| Pentose | Phospho-Ketolase | 2 |
| Pentose | Embden-Meyerhof + pentose cycle | 1.67 |

acetyl-CoA. Such organisms are the yeasts and the lactobacilli. The ATP yields from the butyric acid fermentation by clostridia, from the propionic acid fermentation by propionibacteria, and from the mixed acid fermentations by enterobacteria cannot yet be evaluated owing to the intermediate formation of high-energy acetyl compounds from pyruvate.

The fate of these acetyl compounds varies. Reduction to the corresponding alcohol involves the utilization of a high-energy bond (24). Conversion to the free acid may be coupled with ATP synthesis with acetokinase. Conversion of 2 moles of acetyl CoA to butyryl CoA, on the other hand, involves the utilization of only one high-energy bond, leaving one bond available for ATP synthesis. Despite this uncertainty it is clear that, if hexose is broken down by the Embden-Meyerhof pathway, these organisms will have a net gain of more than 2 moles ATP/mole hexose fermented.

Certain generalizations are possible from the facts so far presented. First, the substrates of all the cleavage enzymes are derivatives of ketoses. It is of interest in this respect that Doudoroff and his colleagues (32, 33) have found that *Pseudomonas saccharophila* metabolizes D-galactose via 2-keto-3-deoxy-D-galactonic acid and D-arabinose via 2-keto-3-deoxy-D-arabonic acid. The former intermediate is phosphorylated with ATP and the product, which has not as yet been isolated, is converted to pyruvate and triose-P. The latter compound is cleaved in the presence of DPN to yield pyruvate and glycollate (Equation 8).

$$\text{2-keto-3-deoxy-D-arabonic acid} + DPN^+ \rightarrow DPNH + \text{pyruvate} + \text{glycollate} \quad 8.$$

These reactions have not as yet been shown to occur in anaerobic systems, but it should not be forgotten that 2-keto-3-deoxy-6-phosphogluconate was first discovered as an intermediate in the oxidation of 6-phosphogluconate by *P. saccharophila* and only subsequently found to be involved in fermentation.

The second generalization is that if cleavage of hexose is preceded by one or more oxidation reactions as in the Entner-Doudoroff pathway, or in the *Leuconostoc* fermentation then the yield of ATP will be 1 mole/mole of hexose. On the other hand, if cleavage occurs prior to oxidation as is the case in the Embden-Meyerhof sequence and in the fermentation of pentose via the phosphoketolase pathway, then the ATP yield is 2 moles/mole substrate fermented. Even when pentoses are first converted to hexose by the pentose cycle the yield of ATP is 1.67 moles. This emphasizes the fact that in the oxidation of hexose, the opening of the ring of the lactone, the primary product of the oxidation, does not appear to be coupled with phosphorylation.

A further point which emerges is that the anaerobic formation of alcohols from acids is a wasteful process. This is true whether we are considering the formation of ethanol by *L. mesenteroides* or *E. coli*, or the formation of ethanol and butanol by *Clostridium butylicum*.

In the reactions discussed so far, it is assumed that the substrate level

phosphorylations supply all the ATP. Kaplan (34) has suggested that electron transfer between DPNH and certain flavoproteins or between pairs of flavoproteins could, in theory at any rate, be coupled with phosphorylation. In a majority of the anaerobic systems which have been examined, there is no indication that such reactions occur. When, however, the *Clostridium kluyveri* system is considered it becomes clear that hitherto unknown energy-yielding reactions must occur. *C. kluyveri* converts a mixture of ethanol and acetate to butyrate, caproate, and hydrogen. The postulated mechanism accounts very well for the formation of these products but, unfortunately, there is no net gain of ATP [Barker (10)]. A key reaction in this fermentation is the oxidation of DPNH by crotonyl CoA which is catalysed by the butyryl dehydrogenase.

$$\text{Crotonyl CoA} + \text{DPNH} \rightarrow \text{Butyryl CoA} + \text{DPN}^+ \qquad 9.$$

Although the butyryl dehydrogenase of *C. kluyveri* has not so far been purified, the corresponding enzyme of animal tissues is a flavoprotein (35). Barker (10) has pointed out that the free energy released in Reaction 9 is about 18 kcal., more than enough for the synthesis of 1 ATP from $P_i$ and ADP, and he has made the suggestion that such a coupling, in fact, occurs.

*Oxidative phosphorylation.*—Pinchot (36, 37) has published the results of further experiments with his preparation from *Alkaligenes faecalis*. Using hexokinase plus glucose as an ATP trap it was found that this preparation coupled the oxidation of DPNH with phosphorylation. The preparation was separated into three components: an insoluble particulate factor; a soluble, heat-labile factor; and a soluble, heat-stable factor. All three were required for oxidative phosphorylation. The heat-stable factor was purified and activity was associated with the polynucleotide fraction. Any treatment which destroyed the polynucleotide also destroyed the activity. Synthetic polyadenylic, polyguanylic, polyuridylic, and polycytidylic acids could all replace the heat-stable factor in the test system. Control experiments appear to have eliminated the possibility that ATP was synthesized indirectly as a result of a preliminary phosphorylation of the polynucleotides which would yield nucleotide diphosphates. DNP had a variable effect on phosphorylation owing, it was found, to the fact that polynucleotide reversed the inhibitory action of DNP. These results suggest that polynucleotides play a part in oxidative phosphorylation. It is also possible to explain the effect of these compounds if the preparation contained an inhibitory compound or compounds, the action of which is reversed by polynucleotides.

In contrast to mitochondria from animal tissues, bacterial preparations have rather low P/O ratios, usually less than 1.0. Brodie & Gray (38), however, have obtained preparations from *Mycobacterium phlei* and *Corynebacterium creatinovorum* with P/O ratios greater than 1.0. Oxygen uptake was measured manometrically and ATP formation was followed with the aid of the hexokinase trapping system. Most of the experiments reported were carried out with preparations from *M. phlei* which oxidized succinate,

2-ketoglutarate, fumarate, malate, pyruvate, and 3-hydroxybutyrate, and in all cases oxidation was coupled with phosphorylation. DNP, 2-nitro-4-amino phenol, azide, arsenate, methylene blue, gramicidin, thyroxine, and triiodothyronine all inhibited phosphorylation. These preparations consisted of a particulate and a soluble fraction, both of which were essential for phosphorylation. Brodie, Weber & Gray (39) have found that when both the supernatant and particle fractions were irradiated at 360 mμ, respiration and phosphorylation were inhibited. The addition of vitamin $K_1$ suspended in the lipide fraction from these organisms completely restored both respiration and phosphorylation. It was also found that irradiation of the vitamin $K_1$-lipide suspension destroyed the activity, from which the conclusion was drawn that the loss of activity on irradiation of both particles and supernatant resulted from the destruction of vitamin $K_1$. Addition of flavin mononucleotide restored the oxygen uptake of irradiated preparations but had no effect on phosphorylation. The supernatant fraction contained a cytochrome-*c* reductase which was markedly stimulated by menadione (2-methyl-1:4-naphthoquinone), (40). The DNP-sensitive component is in the supernatant fraction (41) and Wosilait & Nason (42) have noted that the menadione reductase is sensitive to DNP.

Hartman, Brodie & Gray (43) have also obtained active preparations from *Azotobacter agile*. This system is more unstable than that from *M. phlei*, but, if both ATP and $Mg^{++}$ were added during the course of preparation, the extracts were stabilized. Despite these precautions however, the P/O ratios were lower than those obtained with *M. phlei*. Respiring extracts of *Azotobacter vinelandii* have been prepared by Bruemmer *et al.* (44) and a particulate fraction obtained which contained the respiratory system and oxidized both DPNH and succinate. Tissières, Hovenkamp & Slater (45) fractionated extracts of *A. vinelandii* into large particles, small particles and supernatant. The small-particle fraction of Bruemmer *et al.* (44) had greater respiratory activity than the large-particle fraction; it also gave greater P/O ratios than the large-particle fraction with both succinate and DPNH. The P/O ratios were less than those obtained by Brodie & Gray (38) with their preparation from *M. phlei* and did not exceed 0.87. The *Azotobacter* preparations as a whole seem to be more resistant to DNP than do those from *M. phlei*, (38, 45 to 48).

An attempt has been made by Klungsöyr, King & Cheldelin (49) to study oxidative phosphorylation in washed cell suspensions of *Acetobacter suboxydans* with either glycerol or glucose as the substrate. Very thick cell suspensions were required in order to obtain enough material for the phosphate estimations and this made it necessary to measure the oxygen uptake separately on a diluted (1:4) suspension. On adding substrate, inorganic phosphate disappeared and by keeping the time very short (3.5 seconds), it was possible to obtain a figure for the rate of phosphate uptake from which the P/O ratio could be calculated; in this way, values of *ca.* 0.3 were obtained. The changes observed in inorganic phosphate were very small. When

labelled phosphate was added to suspensions the intracellular pyrophosphate rapidly became labelled and the final specific activity was about half that of the phosphate; ATP, on the other hand, became labelled more slowly. A particulate preparation of *A. suboxydans* has been prepared (50) which contains the cytochrome system and which oxidizes a variety of substrates. On treatment with deoxycholate the cytochrome system was solubilized, but the preparation so obtained oxidized only glucose and galactose.

The microbial preparations so far obtained do not show either the constancy or the high P/O ratios of mitochondria from animal tissues. Whether these low P/O ratios reflect the full potentiality of the system or whether they are an indication of the difficulty of making the preparations is not as yet clear. The microbial systems are complicated by the fact that the cytochromes vary almost from organism to organism [Smith (51)]. It is to be hoped that these studies will continue to be pursued actively, for accurate information on ATP yields in aerobic organisms will be of immense use to those working on bacterial growth *vide infra.*

*Growth yields.*—One fact often overlooked by those interested in reaction mechanisms is that the energy made available by the reactions they study is used by organisms for growth. Monod (52) showed that the dry weight of aerobic organisms produced was proportional to the amount of substrate supplied. Because, at present, there is no reliable assessment of the energy yield from oxidative phosphorylation in bacteria, it has not been possible to follow up this work. Organisms with an anaerobic metabolism offer better opportunities for study and Gunsalus and his colleagues were the first to exploit these (15, 19). In their first experiments (19), these workers compared the molar growth yields (μg. dry wt. of cells/μmole of substrate) of *Streptococcus faecalis* and *Leuconostoc mesenteroides* grown on glucose; and observed that the former value was significantly higher than the latter. They used this observation to support their argument that *L. mesenteroides* ferments glucose by a pathway different from that used by *S. faecalis,* and one which yields less energy. It has subsequently been established that, whereas *S. faecalis* ferments glucose by the Embden-Meyerhof pathway and thus obtains 2 moles ATP/mole of glucose, *L. mesenteroides* ferments glucose via pentose and the phosphoketolase pathway, thereby obtaining 1 mole ATP/mole of glucose. Sokatch & Gunsalus (15) measured the molar growth yields of *S. faecalis* grown on glucose and gluconate and obtained values of 19.6 and 17.6 μg. dry wt. cells/μmole, respectively, and the computed ATP yield from glucose and gluconate are 2 and 1.33, respectively (Table I).

Bauchop (53) has extended these studies. He measured the molar growth yields of *S. faecalis* grown on glucose and ribose and obtained figures of 22 and 20, respectively. The value for glucose is in good agreement with that obtained by Sokatch & Gunsalus (15) and the value for ribose is in keeping with the fact that fermentation of pentoses by the phosphoketolase route yields 2 ATP/mole pentose. In the course of these experiments it was found that, in the presence of glucose, the addition of arginine increased the growth

of *S. faecalis* and calculation of the yield of cells due to arginine gave a value of 10 μg. cells/μmole arginine. This is about half the value obtained for glucose and it is tempting to suggest that this reflects the fact that when arginine is broken down by the arginine dihydrolase system of *Streptococcus faecalis,* 1 μM ATP is synthesized/mole arginine decomposed [Knivett (54)], i.e., half that from glucose. Molar growth yields of *Saccharomyces cerevisiae* grown anaerobically on glucose were also measured and a value of 21 μg. was obtained. The figures for *Propionibacterium pentosaceum* grown on glucose, lactate, and glycerol were 37, 7.7, and 20 μg./μM, respectively. It will be noted that the value for glucose with this organism is considerably greater than the corresponding values obtained with *S. faecalis* and *S. cerevisiae,* and may reflect the fact that propionibacteria have an additional energy course, possibly the conversion of pyruvate to acetate which could be coupled with the synthesis of ATP.

Too few organisms have so far been examined to permit reaching any but the most tentative conclusions. But, in general, it is not going beyond the facts to state that the weight of cell material synthesized during the anaerobic growth of both *S. faecalis* and *S. cerevisiae* is proportional not only to the substrate but also to the computed yields of ATP.

A valuable review of the energy transformations in living matter has been published by Krebs & Kornberg (55).

## Polysaccharides

*Cellulose synthesis.*—Cellulose is produced by both *Acetobacter xylinum* and *Acetobacter acetigenum,* and these two species have been used in a number of laboratories to study cellulose synthesis. Walker & Wright (56) grew *A. acetigenum* on a glucose-lactate medium and found β-cellobiose, β-cellotriose, and β-cellotetraose in the culture filtrate; cellobiose and cellotetraose were isolated as the tetra-acetates. Spots corresponding to fructose, fructose oligosaccharides, and sugar phosphates were also found on chromatograms of culture filtrates. The fructose was found after 24 hr. incubation and cellobiose after 2 days, and the latter was present for the remainder of the incubation period (10 days). The origin of the fructose is obscure but samples of the culture medium taken prior to inoculation do not appear to have been examined.

Steel & Walker (57) extended these observations to *A. xylinum* and *A. xylinum* var. *africanum* and detected both cellobiose and cellotriose in culture filtrates of both organisms. No oligosaccharides were detected in culture filtrates from celluloseless mutants of these two organisms and of *A. acetigenum,* and the authors cite this as evidence that oligosaccharides are intermediates in the synthesis of cellulose. The origin and properties of the mutants are obviously a matter of some importance. Schramm & Hestrin (58) observed a few years ago that when *A. xylinum* was grown in a shaken-liquid medium, the appearance of the culture changed with time. At first the culture consisted of large clumps of cells embedded in cellulose

suspended in the clear medium but, after 3 days, growth became diffuse. After a number of transfers on the same medium incubated under the same conditions, they were able to isolate from the culture, strains which had lost the ability to form cellulose but which were said to resemble the wild type in all other respects.

The isolation of the mutants used by Steel & Walker (57) is described by Steel & Walker (59) and a detailed study of the properties of the isolates was reported in a subsequent paper (60). Like Schramm & Hestrin (58), Steel & Walker took the appearance of diffuse growth as a first indication of the development of mutants; they used a yeast extract-ethanol medium in place of the yeast extract-peptone-glucose medium of Schramm & Hestrin (58). In all, some 26 mutants were examined by Steel & Walker (60). These mutants resembled the parent cultures insofar as they were motile, catalase-positive, Gram-negative rods. On the other hand, not only had they lost the ability to form cellulose, but they were now incapable of either growing on ethanol or producing gluconate from glucose. Of the 21 strains tested, 17 had acquired the ability to grow on an amino acid medium. Of 18 mutants tested, 14 had gained the ability to produce acid from sucrose and fructose and all 26 mutants were now proteolytic. In addition to this, gross changes in colonial morphology and growth rate occurred. The authors (60) do not appear to have considered the possibility that they were dealing with contaminants although they do remark—"Apart from minor differences, the mutant organisms bear a resemblance to the description given, for *Pseudomonas geniculata* in *Bergey's Manual of determinative Bacteriology!*" It would seem necessary to interpret these experiments (57) with caution.

Mutants of *Acetobacter xylinum* and of *Acetobacter pasteurianum* have been isolated by Shimwell (61, 62, 63). The celluloseless mutants of *A. xylinum* appeared to be identical with *Acetobacter mesoxydans* and the mutants of *A. pasteurianum* which were characterized by the loss of the ability to form starch, could not be distinguished from *Acetobacter rancens*.

Cellulose production by growing cultures of *A. xylinum* has also been investigated by Greathouse and his colleagues [Minor *et al.* (64); Minor, Greathouse & Shirk, (65); Greathouse (66, 67)]. The approach here has been to supply glucose, labelled in various positions, as the energy source, and to examine the distribution of $^{14}C$ in the products. The $CO_2$ produced was collected and counted and, at the end of growth, the cellulose was isolated and hydrolyzed to glucose which was then degraded to establish the distribution of the isotope. There was a certain amount of randomization when glucose-1-$^{14}C$ and glucose -2-$^{14}C$ were the substrates; in the case of glucose-6-$^{14}C$, the randomization was almost negligible. Some 76 per cent of the isotope of glucose-1-$^{14}C$ added appeared as $CO_2$ [Minor *et al.* (64)] and 0.55 per cent in the polymer, whereas in the case of glucose-2-$^{14}C$ the figures were 42 per cent and 10 per cent, respectively [Minor *et al.* (65)]. The fact that there was only a small amount of randomization found suggested to Greathouse (66) that the enzymes of the pentose cycle do not

play a very significant part in the metabolism of glucose by this organism.

Hestrin and his colleagues have attacked the problem on a broader front. A much needed micro-method for the estimation of cellulose was first developed [Schramm & Hestrin (68)]. The second step was to prepare both washed cell suspensions and dried cells of *A. xylinum* free from cellulose but which retained the ability to form cellulose [Hestrin & Schramm (69)]. Freeze-dried preparations containing some 10 per cent viable cells formed cellulose immediately on adding glucose (0.25 μM glucose polymerized/mg./hr.) and some 26 per cent of the added glucose was polymerized. The smooth suspension gradually changed to a collection of large, cellulose-containing bodies dispersed in clear medium and the appearance was similar to that of cultures grown on a shaker (58). The polymer, which had all the properties of cellulose, surrounded the cells and none was found inside the cells. Oxygen was essential for the polymerization. Schramm, Gromet & Hestrin (70) studied the polymerization process in more detail using both washed cell suspensions and the freeze-dried preparations. Washed cells formed cellulose from glucose, fructose, gluconate, 2-ketogluconate, 5-ketogluconate, glycerol, and dihydroxyacetone, but not from the following intermediates of the tricarboxylic acid cycle: succinate, fumarate, malate, oxalacetate, pyruvate, acetate, and 2-ketoglutarate. The freeze-dried preparations had lost the ability to form cellulose from 2-ketogluconate and 5-ketogluconate nor were they able to utilize glucose-1-P and glucose-6-P. On the other hand, both cellobiose and maltose were converted to cellulose. Oxygen could not be replaced by a variety of H-acceptors and ATP.

Glucose was rapidly oxidized to gluconate and, although initially glucose was more rapidly converted to cellulose than was gluconate, the rate of polymerization soon fell to that obtained with gluconate. The action of the usual inhibitors was also studied. Arsenite inhibited the formation of cellulose and its action was reversed by orthophosphate. DNP inhibited cellulose synthesis and, at the same time, increased the yield of ketogluconate. It was also found that acetate and pyruvate (50 m*M*) inhibited cellulose formation in contrast to the observation of Minor *et al.* (64) who found that, while acetate was not used by *A. xylinum* it promoted cellulose formation by growing cells. In all, the results of these experiments suggested that the polymerization process involves a phosphorylation step.

The enzymes present in cell-free extracts of *A. xylinum* prepared either with the Mickle disintegrator or by grinding with alumina were examined by Gromet, Schramm & Hestrin (71). Extracts prepared by either method were unable to make cellulose from any of the substrates tested (glucose, α-glucose-1-P, β-glucose-1-P, glucose-6-P, and uridine diphosphoglucose). When reinforced with a carrier, phenazinemethochloride, the extract oxidized glucose to gluconate; supplementing this system with DPN resulted in the oxidization of the gluconate to ketogluconate, chiefly 2-ketogluconate, and when this system was still further reinforced with ATP, $CO_2$ was produced. The following enzymes were demonstrated in the extracts: gluco-

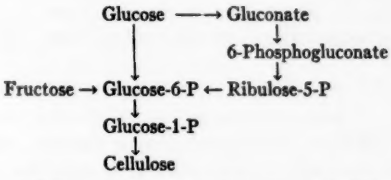
kinase, gluconokinase, phosphoglucomutase, the enzymes of the pentose cycle, and the enzyme discovered by Schramm & Racker (23) which cleaves fructose-6-P to acetyl phosphate and erythrose-4-P.

Schramm, Gromet & Hestrin (72) have studied cellulose synthesis by washed cell suspensions of *A. xylinum* using glucose-1-$^{14}$C, glucose-2-$^{14}$C, glucose-6-$^{14}$C and fructose-1-$^{14}$C. The specific activity of the glucose isolated from the cellulose was expressed as a percentage of the specific activity of the initial glucose and in this way an index ($K$) was obtained of the incorporation of a given glucose carbon atom into the polymer. In the case of glucose-1-$^{14}$C most of the isotope was found in the $CO_2$ and $K = 7$ to 11 per cent; glucose-2-$^{14}$C gave a $K = 78$ to 91 per cent, and with glucose-6-$^{14}$C, $K = 96$ to 100 per cent. In contrast to glucose-1-$^{14}$C, fructose-1-$^{14}$C gave a $K = 35$ to 40 per cent. These results suggest that most of the $CO_2$ produced from glucose by washed cell suspensions is derived from $C_1$. The glucose residues isolated from cellulose produced when glucose-2-$^{14}$C was the substrate were degraded and 47 per cent of the activity was found on $C_1$ and the remainder of the activity was distributed between $C_2$, $C_3$, $C_4$, and $C_5$; $C_6$ was inactive. In the case of glucose-6-$^{14}$C the isotope was found solely on $C_6$ of the monomer.

The results of the experiment with glucose-2-$^{14}$C differ from those obtained by Minor *et al.* (65) who worked with growing cultures. The cellulose formed by growing cells contained approximately 60 per cent of the isotope in $C_2$ of the glucose residues and relatively little (15 per cent) was found in $C_1$. Both groups agree on the fate of $C_6$ of the substrate for neither growing cultures nor washed suspensions randomize this carbon atom and the specific activity of the cellulose-glucose was the same as that of the substrate-glucose. It will be recalled that Greathouse (66) concluded that the pentose cycle plays little part in the metabolism of glucose by growing cultures of *A. xylinum*. In contrast to this conclusion, it would appear that washed cell suspensions oxidize glucose to gluconate which, after phosphorylation, is further metabolized via the pentose cycle. Fructose is not oxidized directly and this is said to explain the relatively large value for $K$ with fructose-1-$^{14}$C, for it is suggested that fructose is metabolized via fructose-6-P and glucose-6-P and glucose-1-P. The pathways proposed by Greathouse (66) and by the Israeli microbiologists are given in Figure 1. It may well be that growing cells behave differently from washed cell suspensions but, at the same time, the scheme put forward by Hestrin and his colleagues is more in keeping with the enzymic constitution of the organism.

The work so far discussed does not give any indication of the mechanism of polymerization. During the past year, cell-free extracts of *A. xylinum* capable of forming cellulose, have been prepared by Greathouse (67), Colvin (73), and Glaser (74). Greathouse (67) prepared extracts by grinding dried cells with powdered glass. The extract was incubated in what amounted to a complete growth medium, viz., 1 per cent glucose, 2 per cent yeast extract, and 0.1 per cent $KH_2PO_4$. This mixture formed cellulose when ATP was

added and the amount of cellulose increased with the amount of ATP (up to a final ATP concentration of 1.6 per cent!). The pH optimum was 8.5. A requirement for ATP for the conversion of glucose to cellulose by extracts was




Cellulose formation after Schramm *et al.* (72)

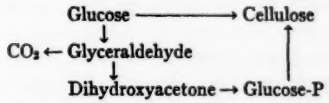



FIG. 1. Cellulose formation after Greathouse (66)

also demonstrated by Colvin (73). Glaser (74) working with a particulate fraction prepared by disrupting *A. xylinum* in a Raytheon sonic disintegrator has found that both uridine diphosphoglucose and a soluble cellulose dextrin are essential for cellulose synthesis. The experiments were carried out with glucose-labelled uridine diphosphoglucose and from the hydrolysis products of the cellulose, both glucose and cellobiose were isolated as crystalline compounds and counted. The major steps in the synthesis would thus appear to be:

Glucose → Glucose-6-P → Glucose-1-P → Uridine diphosphoglucose → cellulose 10.

Presumably the dextrin acts as a primer and this may explain why Gromet *et al.* (71) were unable to demonstrate cellulose synthesis when their preparations were incubated with uridine diphosphoglucose alone.

The site of formation of cellulose in *A. xylinum* is still something of a mystery. Colvin, Bayley & Beer (75) have followed the process with the electron microscope using suspensions of cellulose-free *A. xylinum*. Within 2 min. of adding glucose, fibrils of crystalline polymer about twice as long as the cells appeared. The fibrils appeared to grow by deposition of material at the distal end, and Colvin *et al.* suggest that polymerization is an extracellular process. At no stage were the authors able to detect the presence of amorphous cellulose. The appearance of cellobiose and other β-oligosaccharides in culture filtrates could be considered to support this view, since extracellular synthesis would presumably require a supply of glycosidic bonds at the site of synthesis; it may be significant that Hestrin & Schramm (69) found that their preparations made cellulose from cellobiose and maltose. As against this, is the fact that not one of the postulated intermediates

was converted to cellulose when added to suspensions of either whole cells or freeze-dried preparations which were capable to forming cellulose from glucose [Schramm *et al.* (70); Gromet *et al.* (71)]. If synthesis does occur at the distal end of the fibril, then one would expect to find the necessary enzymes in the culture filtrate, but there are as yet no reports of this. It is clear that more experiments are required to settle this most interesting point.

*Cellulose breakdown.*—The microbial breakdown of cellulose raises a question which is, in fact, common to the breakdown of all insoluble substrates, namely, whether the degradation is brought about by enzymes secreted into the medium (exo-enzymes) or whether the enzymes responsible are bound to the organism, in which case the organisms must be in intimate contact with the substrate. In the case of molds, e.g., *Myrothecium verrucaria* which has received considerable attention recently, the enzymes are found in the culture filtrates. In the case of the anaerobic rumen bacteria [Sijpesteijn (76); Hungate, (77); Baker & Harris (78)] and the Cytophagas [Winogradsky (79); Stanier (80)], it would appear that during growth the organisms adhere closely to their substrate and no cellulolytic enzymes appear in the medium. Halliwell (81) fractionated sheep rumen liquor in which cellulose is rapidly fermented, by centrifuging at 20,000 *G* and found that all the cellulolytic activity was associated with the pellet which contained the bacteria and that the bacteria-free supernatant was inactive. Sijpesteijn (76) maintained her cultures of *Ruminococcus flavefaciens* on strips of filter paper suspended in tubes of agar which had been seeded with the organism. Initially, growth was restricted to the filter paper but, in old cultures, minute satellite colonies appeared in the agar around the digested areas of the filter paper, suggesting that diffusable hydrolysis products, capable of supporting the growth of the organism, were being formed and this in turn suggests the production of an exo-enzyme; it is significant that this organism grows upon cellobiose. None of these experiments is conclusive since, on the one hand, the appearance of cellulases may result from the autolytic release of what was originally an intracellular enzyme and, on the other, a failure to detect cellulolytic activity in a culture filtrate may arise from the absorption of the enzyme on the insoluble substrate.

A second problem is concerned with the nature of the enzymic attack on cellulose. The enzymes which have so far been described are hydrolytic. McBee and his colleagues have demonstrated the presence of a cellobiokinase and a cellobiose phosphorylase in extracts of *Clostridium thermocellum*, [82, 83, 84); see also Crook & Stone (85)]. Halliwell (81) has discussed the merits of the various methods of assay of cellulase activity and particularly the significance of the results obtained with the more common substrates used for this purpose, e.g., carboxymethylcellulose, cellodextrin, hydrocellulose, precipitated cellulose, and native cotton fibres. When dealing with an insoluble substrate the surface area exposed to the enzyme will

be an important factor in comparing activities, as will the presence of a greasy coating. Halliwell (86) studied cellulolytic enzymes from two sources—the very mixed population of bacteria obtained by centrifuging and the organisms which developed when a cellulose-containing medium was inoculated with sheep rumen contents. The following substrates were attacked by the bacteria: cotton fibre, cellulose powder, swollen cellulose powder. Butanol extracts of mixed rumen bacteria which slowly attacked cellulose were far more effective on carboxymethylcellulose. The cellulase of the organisms which developed in the enrichment cultures could be extracted by grinding with alumina and these preparations were more active against cellulose powder than against carboxymethylcellulose. The cellulase from culture filtrates from *M. verrucaria* was similar to that prepared by alumina grinding of the cellulose bacteria.

The discussion on the number of enzymes which are required for the breakdown of cellulose continues. Halliwell (86) thinks that his results are best explained in terms of a multiplicity of enzymes. Grimes, Duncan & Hoppert (87) have fractionated the proteins in culture filtrates of *M. verrucaria* by electrophoresis and have obtained a number of discrete fractions showing cellulolytic activity. Sison, Schubert & Nord (88) have prepared a cellulolytic enzyme from the mold *Poria vaillontii.*

The factors controlling the production of cellulase by *Trichoderma viride* have been examined by Mandels & Reese (89). The enzyme, which is present in the culture filtrates, was estimated using carboxymethylcellulose as substrate [cf. Halliwell (81)]. The ability of a large number of compounds both to support the growth of the organism and to promote cellulase formation was examined; these included pentoses, hexoses, polyhydric alcohols, disaccharides, sugar acids, polysaccharides, and proteins. The enzyme was produced only when the organism was grown on cellulose, modified cellulose, cellobiose, lactose, and glucose and of these lactose and cellulose were the most effective. The authors suggest that cellulase is an inducible enzyme. The fact that growth on glucose led to the production of the enzyme (about 400 per cent as effective as on cellobiose and about 20 per cent as effective as on lactose) would seem to argue against this suggestion. However, the authors point out that no cellulase appeared in the medium until after all the glucose had been utilized and that a high initial glucose content was necessary. These effects could be explained if an inducer, possibly a glycoside, was formed from glucose during the course of its metabolism. Calcium and trace metals particularly $Co^{++}$ and $Zn^{++}$ appear to be necessary for enzyme formation. It would seem desirable to extend this work to a study of the intracellular cellulases if such exist.

*Other polysaccharides.*—Amino sugars and glucuronic acid residues are frequently found in microbial polysaccharides and the discovery of uridine derivatives of acetyl-*N*-glucosamine (90) and of glucuronic acid (91, 92) has led to an examination of the role, if any, of the uridine pyrophosphoglycosyl compounds in the synthesis of bacterial polysaccharides. The role of

uridine diphosphoglucose in cellulose synthesis has already been referred to (74). The synthesis of Type III pneumococcus polysaccharide, which is made up of units of glucuronoside-1-β-4-glucose is being studied by Smith and her colleagues. In their first paper [Smith, Mills & Harper (93)] they report on the amounts of the uridylic acid derivatives found in both capsulated and noncapsulated strains. The major components were uridine diphospho-*N*-acetylglucosamine (35 per cent) and uridinediphosphoglucuronic acid (30 per cent), the remainder consisting of uridine diphosphoglucose, UTP, UDP, and UMP in more or less equal amounts. The authors do not give the absolute amounts of these compounds per unit weight of organisms and it is thus difficult to assess the significance of the presence of these compounds, particularly in the noncapsulated forms. The capsular polysaccharide does not contain acetylglucosamine and the authors suggest that the presence of uridine diphospho-*N*-acetylglucosamine reflects the presence of a somatic polysaccharide which contains this compound. Smith *et al.* (94) have partially purified a polysaccharide from a noncapsulated strain of Type II pneumococci which contains acetyl-*N*-glucosamine and it may well be justified to assume that this somatic polysaccharide is common to all pneumococcal types. The capsular polysaccharide of Type I pneumococci contains galacturonic acid residues and it is of considerable interest that Smith, Mills & Harper (95) have detected uridinediphosphogalacturonic acid in this organism.

The enzymes of pneumococci concerned with the metabolism of uridine compounds have also been studied. Smith, Mills & Harper (93) prepared extracts of noncapsulated pneumococci derived from a Type III and demonstrated the presence of uridyltransferase, nucleoside diphosphokinase, hexokinase, glucose-6-P dehydrogenase, and phosphoglucomutase. They were unable to prepare active extracts from the capsulated strain of Type III, thus making more difficult the task of studying the enzymes of this organism. However, they report that, on adding $^{32}$P-labelled pyrophosphate and uridine diphosphoglucose to washed suspensions of the capsulated form they were able to isolate from the reaction mixture labelled UDP and UTP; the amounts formed were not given. This experiment, the authors claim, shows the presence of uridyltransferase.

$$\text{Uridine diphosphoglucose} + \text{PP} \rightleftharpoons \text{UTP} + \text{Glucose-1-P} \qquad 11.$$

It may well be true that the organism contains this enzyme but the labelled UDP and UTP could equally well have arisen, either by a direct interaction of the pyrophosphate with intracellular UTP or, indirectly, through the activity of the powerful pyrophosphatase which the authors claim is present and which would form phosphate from pyrophosphate. Unfortunately, the authors have failed to include in their paper the results of control experiments with labelled pyrophosphate alone. It would also be in-

teresting to know whether, in fact, the uridine diphosphoglucose added actually penetrated the capsulated cells as instances of the penetration of such large molecules are rare. It was also found that when UTP and ADP were added to cell suspensions, ATP and UDP were formed and this, the authors claim, shows the presence of nucleoside diphosphokinase.

A general review of the synthesis of hyaluronic acid has been published by Whistler & Olson (96). Cifonelli & Dorfman (97) have examined the uridine derivatives of hyaluronic acid-forming Group A *Streptococcus*. Ion exchange chromatography on Dowex-1-Cl showed the presence of uridine diphospho-*N*-acetylglucosamine, uridine diphosphoglucose, uridine diphosphoglucuronic acid and an unknown compound which the authors suggest is the corresponding derivative of muramic acid, the hexosamine present in cell walls and in the "spore peptide" of Strange & Powell (98). Muramic acid was isolated by Strange & Dark (99) as a crystalline compound and the following structure proposed by Strange (100)—3-*O*-α carboxyethyl-D-glucosamine—has now been confirmed by synthesis [Kent (101)]. Park & Strominger (102) have shown that the hexosamine of the Park nucleotides [Park (103)] is muramic acid. Cummins & Harris (104) and Work (105) have discussed the composition of cell walls, and Salton (106) has reviewed the action of lysozyme.

The synthesis of chitin, a polyacetylglucosamine, has been studied by Glaser & Brown (107, 108) using cell-free extracts of *Neurospora crassa*. Uridine diphospho-*N*-acetylglucosamine is the substrate of the enzyme and this could not be replaced by either acetylyglucosamine, acetylglucosamine-1-P, or acetylglucosamine-6-P. Acetylglucosamine speeded up the rate of synthesis from the uridine-substrate but it was not incorporated into the polymer. As in cellulose synthesis [Glaser (74)], a soluble primer, in this case chitodextrin, was required and when labelled chitodextrin was used, the chitin synthesized was labelled. The role of uridine diphospho-*N*-acetylglucosamine in the synthesis of chitin was established using the substrate labelled either on the acetyl or on the glucosamine residues. The synthesis of chitin can therefore be expressed by the equation:

$$\text{UridineDPAG} + (\text{acetylglucosamine})_n \rightarrow \text{UDP} + (\text{acetylglucosamine})_{n+1} \qquad 12.$$

Doetsch *et al.* (109) have studied the formation of a glucose polymer by an anaerobic organism, *Bacteriodes amylogenes*, which they isolated from the rumen of sheep. This organism ferments a wide range of pentoses, hexoses, and disaccharides with the formation of volatile acids; the products from D (+) xylose were acetic, propionic, and butyric acids with a trace of lactic acid. After growth the cells stained blue with iodine due to the presence of glucose polysaccharide. This was formed from all carbohydrates capable of supporting growth, among which were D (+) xylose, D (−) arabinose, L (+) rhamose, L (−) fucose, and xylans. The forma-

tion of a hexose polymer from pentose is a strong indication of the active participation of pentose cycle enzymes and a more detailed study of the enzyme systems present in this organism would be of considerable interest [cf. (15, 28 to 31)].

Glucosan synthesis by *Aspergillus niger* from glucose-1-$^{14}$C and ribose-1-$^{14}$C has been studied by Van Sumere & Shu (110) and the distribution of the isotope in the glucose residues of the polymer can best be explained in terms of the pentose cycle. Loewenburg & Reese (111) state that the fructose polysaccharide of *Acetobacter acetigenum* is a 2:6 levan and that of *Aspergillus sydowi* is a 2:1 fructosan. The factors controlling the formation of fructosanase by a number of organisms and the specificity of these enzymes is also described.

The structure of the levan produced by a species of *Corynebacterium* [Henis (112)] has been investigated by Avigad & Feingold (113). The molecular weight (determined by ultracentrifugation) was $12.5 \times 10^6$, and the basal unit contained 15 fructose units linked 2$\beta$:6 with branching linkages 2$\beta$:1. The culture fluid was found to contain oligosaccharides as well as the levan, some 16 gm. being isolated from 5 l. of the medium. The major component was 1$^F$-$\beta$-fructosyl sucrose. The same trisaccharide is formed by another levan-forming organism, *Aerobacter levanicum* [Feingold, Avigad & Hestrin (114)] and because of this Avigad & Feingold (113) suggest that the *Bacillus subtilis* trisaccharide might also have this structure and not 6$^F$-$\beta$-fructosyl sucrose as suggested by Dedonder & Noblesse (115) and by French & Wild (116). A halophilic organism which forms levan has been reported by Lindeberg (117). Avigad (118) has described a new trisaccharide formed when the levan sucrase of *Aerobacter levanicum* acts upon a mixture of sucrose and lactose, and has shown it to be $\alpha$-lactosyl-$\beta$-fructofuranoside.

The factors controlling the production of an intracellular polysaccharide "glycogen" by *E. coli* have been examined by Holme, Laurent & Palmstierna (119), Holme (120), and Eliasson *et al.* (121). Holme *et al.* (119) record that during the period when "glycogen" was being laid down the molecular weight of the polymer (measured by the light scattering method) steadily increased. Conversely, when conditions were altered and glycogen was consumed, the molecular weight steadily decreased. The effect of generation time and nutritional factors on the amount of the polysaccharide formed was studied by Holme (120). For this purpose *E. coli* was grown in continuous culture with either the nitrogen source (ammonium chloride) or the carbon source (glucose or lactate) as limiting factors; generation time was controlled by the dilution rate. Under conditions of nitrogen limitation both the "glycogen" content of the cells and the "specific rate of glycogen synthesis" increased with decreasing dilution rate; that is to say the longer the generation time, the more polysaccharide was formed by the cells. The results were the same whether glucose or lactate was used as energy source. On the other hand, when growth was limited by the carbon source,

neither the polysaccharide content nor the specific rate of synthesis were influenced by the dilution rate. During the lag phase the "glycogen" content of *E. coli* increased nineteenfold whereas other cell constituents, RNA, DNA, protein-N, increased only 2 to 4 times; Palmstierna & Stjerholm (122) found that $^{14}CO_2$ is incorporated into the "glycogen" of *E. coli*, and the label was found mainly in $C_3$ and $C_4$ of the glucose residues which were approximately equally labelled.

Gary *et al.* (123) have also observed an effect of the amount of nitrogen supplied as casein hydrolysate on polysaccharide synthesis by *Brucella suis*. Increasing the amount of casein hydrolysate reduced the polysaccharide content of the cells. Mixtures of amino acids, particularly those containing DL-valine also inhibited. It would seem that we are dealing here with two phenomena, a nitrogen effect similar to that studied by Holme (120) and the more specific effect of DL-valine.

The continuous culture technique was used by Rogers (124) to study the factors controlling hyaluronidase formation by *Staphylococcus aureus*. A "bactogène" of the type described by Monod (125) was used. When the culture had reached the required density, the flow of medium was started and samples were removed at intervals for assay of hyaluronidase. Despite a constant cell density, the enzyme content of the medium increased steadily for the first 10 to 11 generations and thereafter the ratio mass of cells/units hyaluronidase remained constant for the duration of the experiment. The author, having excluded the possibility that the increased enzyme formation was caused by the selection of a mutant, concluded that the initial low synthetic ability was the result of a nutritional deficiency. Cells from an overnight culture were found to be deficient in thiamine and it was subsequently found possible to suppress hyaluronidase formation by reducing the thiamine content of the medium. Nicotinic acid deficiency had the same effect.

The hydrolysis of pentosans by rumen bacteria has been studied by Howard (126) and by Pazur *et al.* (127). Howard used as substrates the soluble pentosans of wheat flour and of *Rhodymenia palmeta* (rhodymenan). The former, which contains 300 anhydropentose units/mole, is made up of a D-xylopyranoside chain, linked β-1 : 4 with single L-arabofur anoside units along the chain linked at positions 2 and 3. Rhodymenan contains 16 to 17 xylopyranoside units linked mainly β-1 : 4 but with about one linkage in five linked β-1 : 3. The pentosans were incubated with a suspension of sheep rumen bacteria in a bicarbonate-buffered saline and both were completely hydrolysed in *ca.* 18 hr. The arabinose units of the wheat flour pentosan were quickly removed by the enzyme system and the xylose chain was fragmented (mild acid hydrolysis had much the same effect); subsequently, the xylose-containing fragments were completely hydrolysed. Partial hydrolysates, made by stopping the enzyme after a limited incubation period, were found to consist of a mixture of arabinose, xylobiose, xylotriose, and xylotetraose. Partial hydrolysis of rhodymenan yielded xylobiose,

xylotriose, and xylotetraose along with some hitherto unknown xylose oligosaccharides, two of which were isolated and identified as *O*-β-D-xylopyranosyl-(1 → 3)-D-xylopyranose and *O*-β-D-xylopyranosyl-(1 → 3)*O*-β-D-xylopyranosyl-(1 → 4)-D-xylopyranose. Pazur *et al.* (127) used xylan prepared from corn cobs. The rumen contents, which were obtained from an Angus bull, were filtered and stored frozen. Such preparations hydrolysed the xylan and the products, which were analyzed by paper chromatography, contained xylose and xylose oligosaccharides. Markers were prepared by partial acid hydrolysis of the xylan. Careful examination of the photographs of the xylose spots obtained from both acid and the enzymic hydrolysate, suggests that the xylan contained or was contaminated with a pentose with an $R_F$ similar to arabinose. Organisms capable of growing on xylan were isolated [cf. Doetsch *et al.* (109)], and a more detailed description is awaited with interest.

The synthesis of rhamnolipide, the curious compound of L-rhamnose and 3-hydroxydecanoic acid [Jarvis & Johnson (128)] formed by *Pseudomonas aeruginosa* when grown on glycerol or fructose, has been examined by Hauser & Karnovsky (129). Acetate-1-$^{14}$C supplied carbon for the synthesis of the hydroxy acid but not for rhamnose. Glycerol-1-$^{14}$C and glycerol-2-$^{14}$C, on the other hand, were converted into rhamnose and also supplied carbon for the synthesis of 3-hydroxydecanoic acid. Glycerol-1$^{14}$C produced rhamnose labelled equally on $C_1$, $C_3$, $C_4$, and $C_6$; presumably two forms of labelled rhamnose were formed, viz., L-rhamnose-3:4-$^{14}$C and L-rhamnose-1:6-$^{14}$C [cf. Rush *et al.* (130)]. With glycerol-2-$^{14}$C, L-rhamnose-2:5-$^{14}$C was formed. In a brief report Hauser & Karnovsky (131) state that when the enantiomorph D-glycerol-1-$^{14}$C is used the rhamnose isolated from the rhamnolipide was L-rhamnose-1:6-$^{14}$C. Presumably, two glycerols are converted to a hexose which is subsequently dehydrated to give L-rhamnose. L-Fucose also appears to be synthesized directly from hexose. *Aerobacter aerogenes* forms a polysaccharide containing L-fucose, galactose, and a uronic acid and Wilkinson (132), Heath & Roseman (133), and Segal & Topper (134) have studied the synthesis of the L-fucose component of this polysaccharide. The organism was given specifically labelled glucose and the polysaccharide formed was isolated, hydrolysed, and both the glucose and L-fucose components isolated. When glucose-1-$^{14}$C and glucose-6-$^{14}$C were the substrate, the L-fucose was labelled on $C_1$ and $C_6$, respectively, indicating a direct transformation of glucose to L-fucose.

Polysaccharides containing aldoheptoses are turning up with increasing frequency. D-Glycero-D-galactophetose has been isolated from the specific polysaccharide of *Chromobacterium violaceum* (BN) by MacLennan & Davies (135). The polysaccharide from a different strain of *C. violaceum* (NCTC 7917) was found to contain D-glycero-D-mannoheptose or its enantiomorph L-glycero-L-mannoheptose (136), and an as yet unidentified aldoheptose has been demonstrated in the lipopolysaccharide antigen of *Pasteurella septica* (137).

### The Oxidation of Aromatic Compounds

The oxidation of aromatic compounds by microorganisms has been reviewed by Evans (5). Until recently, interest has centered mainly on the breakdown of aromatic amino acids, mandelic acid, and phenolic compounds. The metabolic routes which have been clearly established lead to one of three diphenolic intermediates: catechol (I), protocatechuic acid (VI), or homogentisic acid (XI). Highly specific enzymes exist which split the aromatic nuclei of these compounds to yield *cis-cis*-muconic acid (II), *cis-cis*-β-carboxymuconic acid (VII), or maleyl-(*cis*)-acetoacetic acid (XII), respectively, as the first identifiable aliphatic intermediates. These products are further degraded to give substrates capable of entering the tricarboxylic acid cycle. There is evidence that gentisic acid also undergoes ring cleavage (138, 139) but, as yet, little is known of this reaction. Recent work in the field of aromatic oxidations has been concerned with the further examination of the cleavage enzymes, with the details of the steps following ring cleavage and with the breakdown of more complex aromatic derivatives.

*Ring cleavage.*—Gross, Gafford & Tatum (140) have purified fifteenfold the protocatechuic oxidase of *Neurospora*. As with the *Pseudomonas* enzyme previously studied by Stanier & Ingraham (141) the product is a carboxymuconic acid, probably the *cis-cis*-isomer. No cofactor requirements were observed but later work by Ottey & Tatum (142) has shown that the activity of preparations obtained by ammonium sulphate fractionation, dialysis, fractionation with calcium phosphate gel and further dialysis is increased two or threefold by ferrous ions. Mercurials inhibit the enzyme. The *Neurospora* enzyme is thus brought into line with the other enzymes which require ferrous ions and intact -SH groups for splitting the benzenoid ring (141, 143). With the *Pseudomonas* enzyme, Stanier & Ingraham (141) failed to demonstrate any cofactor requirement after several treatments which gave a seven to eight fold overall purification but Dagley & Patel (144), studying the oxidation of protocatechuic acid by a different strain of *Pseudomonas*, observed a requirement for ferrous ions after a crude extract was fractionated with ammonium sulphate and then dialyzed.

The *Neurospora* enzyme will only use oxygen as the oxidant. This is a third common feature of the enzymes cleaving the benzenoid ring and the experiments of Hayaishi, Katagiri & Rothberg (145) with $^{18}O$ have confirmed that in the case of catechol oxidase at least, the enzyme is a "true oxidase," in that the oxygen atoms appearing in the product are derived from molecular oxygen and not from water. The common properties of these oxidases are also shared by a system splitting the pyridine ring [Behrman & Stanier (146)]. Extracts of a *Pseudomonas* oxidizing nicotinic acid, carry out a reaction analogous to that of homogentisic oxidase in which the nucleus of 2:5 dihydroxypyridine is attacked. The system requires ferrous ions and oxygen [c.f. Pinsky & Michaelis (147)]. A bona fide case where there is no oxygen requirement would seem, however, to be established by the work of Harary (148) who has isolated a *Clostridium*

capable of breaking down nicotinic acid under anaerobic conditions to yield ammonia and carbon dioxide together with acetic and propionic acids.

The inhibition of catechol oxidase from mushrooms by borate has been studied by Yasunobu & Norris (149) who obtained kinetic and spectroscopic evidence that a borate-substrate complex is involved.

*Further metabolism of the cleavage products.*—In the *Neurospora* system the primary product of the oxidation of protocatechuic acid, *cis-cis*-β-carboxymuconic acid (VII) undergoes an intramolecular rearrangement to form a lactone which is subsequently hydrolyzed and decarboxylated to yield the enol form of 3-ketoadipate (IV). The lactonizing enzyme from *Neurospora* has been separated from the other enzymes in the sequence and used to prepare the lactone (140). The identification of the various isomers of β-carboxymuconic acid and its lactones is severely handicapped by the lack of reference compounds whose structure has been proved (150), consequently, the identity of the isolated lactone has not been demonstrated unequivocally. Its physical and chemical properties were examined and compared with those of a racemic lactone prepared by chemical means from a biologically produced carboxymuconic acid. The synthetic lactone was almost certainly (+) (−)-β-carboxy-γ-carboxymethyl-$\Delta^{\alpha}$-butenolide (VIII), and the isolated lactone appeared to be the (−) enantiomorph. The isolated compound was attacked by crude *Neurospora* extracts and half of the synthetic compound was metabolized at a comparable rate. The breakdown of protocatechuic acid by *Neurospora* thus proceeds according to Figure 2b, which is analogous to the pathway established for the oxidation of catechol to 3-ketoadipate (V), by *Pseudomonas* via the muconolactone III [(151, 152), Fig. 2a].

The pathway of conversion of β-carboxymuconic acid to 3-ketoadipate plus carbon dioxide in *Pseudomonas*, however, differs from that in *Neurospora* since Gross, Gafford & Tatum (140) found that extracts of Stanier's *Pseudomonas fluorescens* capable of carrying out the overall reaction did not attack either the synthetic or enzymically produced specimens of lactone VIII. Isotope studies confirmed a difference between the two systems. Labelled protocatechuic acid (produced biologically from labelled glucose) was oxidized to 3-ketoadipic acid with extracts of *Neurospora* and *Pseudomonas*. The 3-ketoadipic acid was subsequently degraded by chemical means. The combined results on the distribution of isotope in the degradation products indicated that in the *Pseudomonas* system the carbonyl group of 3-ketoadipate is derived equally from $C_1$ and $C_6$ of the original protocatechuic acid. In the *Neurospora* system, however, it was derived solely from one of these carbons and from the structure of the known lactone intermediate VIII it was inferred that in this case the parent carbon atom was $C_6$. 3-Ketoadipate with the carbonyl group derived from $C_1$ would be expected if β-carboxymuconic acid were metabolized, not via lactone VIII, but by its isomer, lactone IX (γ-carboxy-γ-carboxymethyl-$\Delta^{\alpha}$-butenolide, Fig. 2b). It would perhaps seem possible to explain the results with *Pseudomonas* by postulating that in this organism the conversion of carboxymuconic acid to

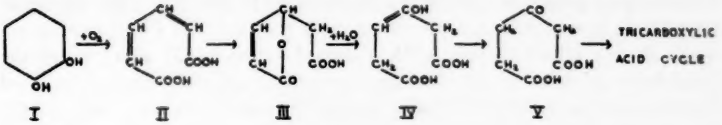



FIG. 2a

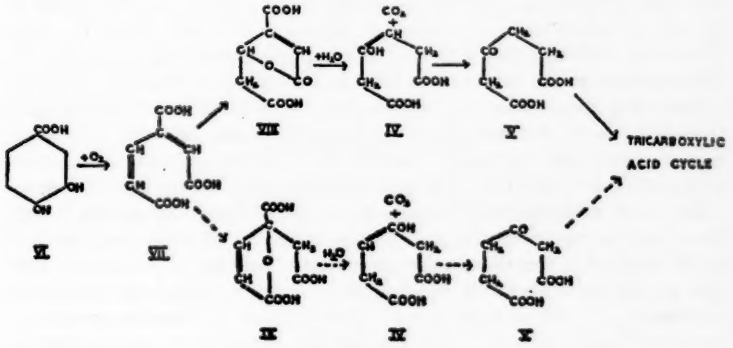
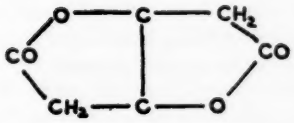



FIG. 2b



X

FIG. 2c

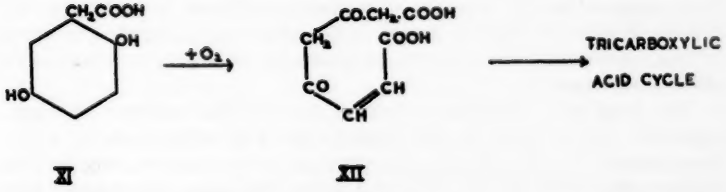



FIG. 2d

3-ketoadipate proceeds equally via lactones VIII and IX. This attractive hypothesis, however, is not consistent with the fact that *Pseudomonas* extracts do not attack lactone VIII. Tatum & Gross (153) also studied the breakdown of labelled catechol by extracts of suitably adapted cells of *Pseudomonas* and obtained results essentially similar to those with protocatechuic acid: the carboxyl group of 3-ketoadipate is derived equally from the two carbons *para* to the hydroxyl groups of catechol.

These results suggest that in *Pseudomonas,* the conversion of muconic acid and carboxymuconic acid to 3-ketoadipate involves a symmetrical intermediate which can be attacked at one of two points giving the same product in each case, but with two different orientations with respect to the carbon atoms of the parent compound. It is tempting to think that the same symmetrical intermediate is involved in the formation of 3-ketoadipate from both these substrates. If so, the dilactone of muconic acid (X) would seem to satisfy the requirements of this intermediate and has, in fact, been prepared chemically (154). The random cleavage of either lactone ring together with nonhydrolytic cleavage of the other could explain the isotope data. Such a mechanism applied to the oxidation of protocatechuic acid would imply that decarboxylation precedes the opening of the lactone ring. The precise point at which decarboxylation occurs is unknown but it does not immediately follow formation of a monolactone of carboxymuconic acid since this would lead to lactone III and this compound is not attacked by extracts of either *Pseudomonas* or *Neurospora* capable of oxidizing protocatechuic acid (140). It would appear possible that in *Neurospora,* hydrolysis of a lactone ring precedes decarboxylation, in which case the presence of the extra carboxyl group in an otherwise symmetrical molecule may exert a directive influence on the hydrolysis and so lead to a unique derivation of 3-ketoadipate from protocatechuic acid.

Dagley & Patel (144) have isolated the end product of the oxidation of protocatechuic acid by extracts of their strain of *Pseudomonas* and found that during this oxidation one mole of oxygen is consumed per mole of protocatechuic acid and no carbon dioxide is evolved. The product contains no phenol or enol groups and the empirical formula $C_7H_6O_6$ suggests that it is a carboxymuconic acid or a carboxymuconolactone. The former possibility is ruled out by the fact that the compound contains only two carboxyl groups for every seven carbon atoms. This is also suggestive of a monolactone, but a dilactone with one labile link would also behave in this way. The compound has an absorption maximum at 276 mμ in contrast to the lactone of Gross, Gafford & Tatum (140) which has negligible absorption at this wavelength. The complete identification of this intermediate is of obvious importance.

The breakdown of protocatechuic acid by cell-free extracts of microorganisms usually proceeds no further than 3-ketoadipate. Kilby (155) demonstrated the quantitative conversion of 3-ketoadipate to succinate by washed cells of *Vibrio* O1 (156) and it has been generally assumed that 3-ketoadipate is split to yield succinate and acetate which then enter the

tricarboxylic acid cycle. In the past year this reaction has been demonstrated in cell-free extracts by Katagiri & Hayaishi (157), so completing the enzymic link-up with the tricarboxylic acid cycle.

These workers grew *Pseudomonas fluorescens* in the presence of tryptophan and obtained extracts which broke down 3-ketoadipate provided sparker amounts of succinyl CoA were added, together with either substrate amounts of CoA or catalytic amounts of CoA plus a system capable of regenerating free CoA from acetyl CoA. The products of the reaction were shown to be succinate and acetyl CoA and the amounts formed were equivalent to the amount of 3-ketoadipate disappearing. The reaction appeared to involve a thiophorase and a thiolase as follows:

$$\text{succinyl CoA} + \text{3-ketoadipate} \xrightarrow{\text{thiophorase}} \text{3-ketoadipyl CoA} + \text{succinate} \quad 13a.$$

$$\text{3-ketoadipyl CoA} + \text{CoA} \xrightarrow{\text{thiolase}} \text{acetyl CoA} + \text{succinyl CoA} \quad 13b.$$

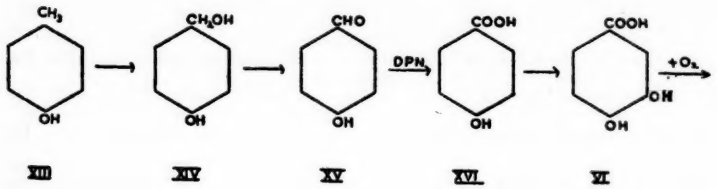
$$\text{3-ketoadipate} + \text{CoA} \longrightarrow \text{acetyl CoA} + \text{succinate} \quad 13c.$$

The thiophorase, which has been purified twenty-fivefold and obtained free of thiolase activity, has been used for the enzymic preparation of a substance having the properties expected of 3-ketoadipyl CoA. In the presence of $Mg^{++}$ and at pH above 8, this compound has a strong absorption at 350 mμ and this property has been used to follow the thiophorase reaction spectrophotometrically in both directions. The thiophorase shows a high specificity; thus, acetyl CoA will not replace succinyl CoA in the forward reaction and malonate, fumarate, oxalate, and acetate will not replace succinate in the reverse reaction. The thiolase was shown to be reversible by the use of radioactive succinate which was incorporated into 3-ketoadipate during the overall Reaction 13c.

The properties of the system are thus ideally suited to the role of producing intermediates for feeding into the tricarboxylic acid cycle since, of the two CoA thioesters produced by the thiolase reaction, acetyl CoA, the true substrate of the tricarboxylic acid cycle, cannot be utilized by the thiophorase, whereas the other, succinyl CoA, can. This, however, gives succinic acid which can still enter the cycle. Succinyl CoA itself is an intermediate in the cycle and would therefore presumably be available in sparker quantities in the intact cell if required. If the tricarboxylic acid cycle enzymes are situated on particles in the intact cell, this explains why the breakdown of aromatics by cell-free extracts usually stops at 3-ketoadipate since, in the absence of the cycle, no other mechanisms may exist for the regeneration of CoA from acetyl CoA and sparker amounts of succinyl CoA may not be available.

*Oxidation of other aromatics.*—The work of Dagley & Patel mentioned earlier was part of a detailed investigation (144) into the bacterial oxidation of *p*-cresol, previously reported by Smith, Jones & Evans (158). Dagley & Patel used a strain of *Pseudomonas* isolated from an experimental plant

for the treatment of effluent from coal-gas works. After growth on *p*-cresol, washed cells of the organism oxidized *p*-cresol (XIII), *p*-hydroxybenzaldehyde (XV), *p*-hydroxybenzoic acid (XVI), protocatechuic acid, citrate, and succinate, but did not oxidize catechol. Freeze-dried cells and extracts of crushed cells were also capable of oxidizing *p*-cresol. With the latter, the rate of oxidation was increased by adding a pink particulate fraction which displayed a typical cytochrome spectrum. Crude extracts oxidized suspected intermediates to the unknown lactone mentioned earlier and a study of these oxidations, which included the isolation and spectroscopic identification of the products, indicated the following reaction sequence:




UNKNOWN LACTONE ⟶ 3-KETO-ADIPATE ⟶ TRICARBOXYLIC ACID CYCLE

FIG. 3

The participation of *p*-hydroxybenzyl alcohol (XIV) in this chain is inferred as it was not tested by Dagley & Patel, but Smith, Jones & Evans mentioned that this compound is oxidized by a soil *Pseudomonas* after growth on *p*-cresol. The ability to hydroxylate *p*-hydroxybenzoic acid which has not been obtained previously in a cell-free system, was lost after precipitation with ammonium sulphate. The ability to oxidize *p*-hydroxybenzaldehyde to *p*-hydroxybenzoic acid is also lost but is restored by DPN; TPN will not replace DPN.

The oxidation of *p*-cresols bearing an extra methyl or hydroxyl group in the nucleus was also studied. Washed cells oxidized these compounds without affecting the substituent groups, to give the corresponding derivatives of *p*-hydroxybenzoic acid, by way of the corresponding aldehydes. When 4-methyl-catechol was the substrate, the acid produced was protocatechuic acid and was oxidized further. In the other cases, the acid was not metabolized and accumulated. It would thus appear that the oxidation of the side chain requires the presence of a *para* hydroxyl group since only the 4-methyl group of 2:4-xylenol and 3:4-xylenol is attacked. Similarly, *p*-hydroxybenzaldehyde is attacked whereas benzaldehyde itself is not. Substitution elsewhere in the nucleus does not prevent oxidation of the side chain, but interferes with hydroxylation of the ring.

The breakdown of polycyclic hydrocarbons by soil organisms was reported some thirty years ago and at that time Tausson (159, 160) and Gray & Thornton (161) isolated organisms capable of utilizing naphthalene (XVII) and phenanthrene (XX) for growth. In recent years, the breakdown of these hydrocarbons has been studied further in several laboratories, using organisms isolated from soil by various enrichment procedures.

Walker & Wiltshire (162) grew an organism resembling the *Bacillus naphthalinicus non-liquefaciens* of Tausson, on a salts-naphthalene medium and isolated from the culture D-*trans*-1:2-dihydro-1:2-dihydroxynaphthalene ("naphthalene diol," XVIII) after two days growth and salicylic acid (XIX) after longer periods. These observations were later extended to three *Pseudomonas* strains and another organism thought to be a *Nocardia* (163). Strawinski & Stone (164) also isolated salicylic acid from cultures of *Pseudomonas* and in a later paper by Murphy & Stone (165), the changes in salicylate concentration and cell numbers were reported. The cell count reached a maximum after 10 hr., then decreased between 10 and 20 hr. to a value near that which obtained after inoculation. After 20 hr. a secondary rise in cell count occurred, comparable in magnitude to the first rise. Salicylate reached a maximum concentration at 15 hr. and thereafter disappeared. The authors liken the growth curve to the "diauxie" effect of Monod (52) and the results suggest an interesting interaction between the organism and an intermediate with apparent bactericidal properties. It is unfortunate that the authors give few experimental details. In particular, the method of counting cells is not described, consequently it is not clear whether the results refer to viable cells only; in addition, no details are given as to how the media or culture vessels (some of which were large) were sterilized; in fact, there is no statement that they were sterilized.

The amounts of salicylate found in all of the above investigations were small compared with the amount of naphthalene used and this led Klausmeier & Strawinski (166) to investigate the conditions affecting salicylate accumulation, using selected strains of *Pseudomonas aeruginosa*. For optimal yields, forced aeration, a pH near 8, a high phosphate concentration and the presence of calcium, iron, and magnesium were required, and under these conditions the amount of salicylate accumulating was comparable with the original naphthalene content of the medium. A third possible intermediate was noted by Murphy & Stone (165), who observed the accumulation in the medium of an orange pigment, especially when iron and magnesium were deficient. This was isolated and shown to be 1:2-naphthoquinone.

Salicylate has been confirmed as an intermediate by studies with washed cells and cell-free extracts. Treccani, Walker & Wiltshire (163) found that suspensions of naphthalene-grown cells oxidized both salicylate and catechol without lag, whereas succinate-grown cells did not. Murphy & Stone (165) obtained similar results and prepared cell-free extracts capable of oxidizing these two compounds; they demonstrated the formation during

these oxidations of a product which gave a positive Rothera test, presumably 3-ketoadipate. 1:2-Naphthoquinone could not be confirmed as an intermediate. It was not attacked by washed cells or cell-free extracts and inhibited the oxidation of other substrates by both growing cells and washed suspensions (163, 165, 167). Napthalene diol was oxidized by washed cells (162) although Murphy & Stone could not detect it in their cultures. Tests with naphthols were either negative or inconclusive; thus, some of the strains of Treccani, Walker & Wiltshire oxidized α- and β-naphthols but concentrations above $10^{-3}M$ were inhibitory and one strain oxidized these compounds even after growth on succinate. 1:2-Dihydroxynaphthalene was oxidized to 1:2-napthoquinone both in the presence and absence of cell-free extract (165).

It thus appears that naphthalene is oxidized via salicylate, catechol, and 3-ketoadipate with naphthalene diol as a possible intermediate (Fig. 4). Murphy & Stone suggest that a second pathway leads to 1:2-naphthoquinone which is not metabolized further and it would seem that both the diol and 1:2-dihydroxynaphthalene may be intermediates in this latter reaction.

Rogoff & Wender (167) have obtained a phenanthrene-oxidizing strain of *Pseudomonas* and have isolated 1-hydroxy-2-naphthoic acid (XXI) from the medium. This compound is oxidized by washed cells as are catechol, salicylic acid, and protocatechuic acid. However, the oxidation of 1-hydroxy-2-naphthoic acid only proceeds to a limited extent, and with the release of a roughly equivalent amount of carbon dioxide. The authors suggest that the immediate product may be 1:2-dihydroxynaphthalene which is oxidized, possibly nonenzymically, to give 1:2-naphthoquinone. It was shown that the latter inhibited endogenous respiration and the oxidation of salicylic acid, when added in amounts equivalent to the carbon dioxide produced from 1-hydroxy-2-naphthoic acid. If 1-hydroxy-2-naphthoic acid can be established as a true intermediate, this would exclude the theory of Tausson (168) [quoted by Rogoff & Wender (167)] which suggests that the central ring of phenanthrene is attacked first, a postulate based on the facts that this ring is the least stable chemically and that *o*-hydroxybenzyl alcohol, *o*-hydroxybenzaldehyde, salicylic acid, and catechol supported the growth of his phenanthrene oxidizers.

Rogoff & Wender (169) have also grown a naphthalene-oxidizing strain on anthracene (XXII) and in this case 3-hydroxy-2-naphthoic acid (XXIII) was isolated from the medium. The pathways at present envisaged for the oxidation of these hydrocarbons are summarized in Figure 4.

Interest in the decomposition of lignin has led to studies of the metabolism of compounds known to be produced by chemical degradation of this substance. Henderson & Farmer (170) isolated a wide range of fungi capable of growing on *p*-hydroxybenzaldehyde, two of its methoxylated derivatives, vanillin (XXIV) and syringaldehyde (XXVII), and ferulic acid (XXVI). Using paper chromatography and spectrophotometry, sub-

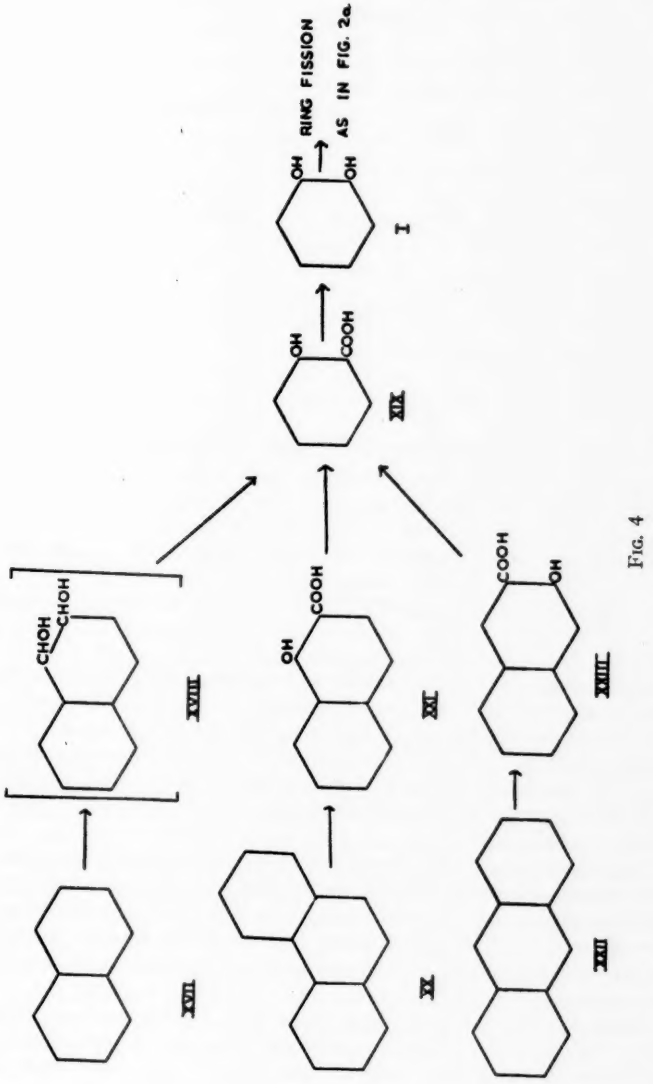

RING FISSION
AS IN FIG. 2a.
OH
OH
I
OH
COOH
XIX
CHOH
CHOH
XVIII
OH
COOH
XX
COOH
OH
XXIII
XVII
XXI
XXII

Fig. 4

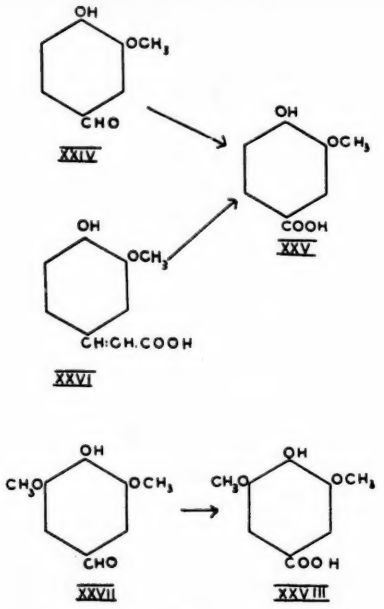



FIG. 5

stantial amounts of vanillic (XXV) and syringic (XXVIII) acids, were demonstrated in cultures utilizing vanillin and syringaldehyde, respectively. Vanillic acid was also detected in cultures growing on ferulic acid. The role of these acids as intermediates in the breakdown of the respective substrates was confirmed by the finding that they were oxidized without lag by fungal preparations only after adaptation to the appropriate substrate [Henderson (171)].

The preparations used in these latter studies were obtained by setting up spore suspensions with suitable media in Warburg manometer flasks under aseptic conditions and then incubating overnight, during which time the spores germinated. These preparations were considered superior to mycelial mats in being more reproducible and more homogenous, and in having a lower endogenous respiration.

Delignification in soil is accompanied by a fall in methoxyl content (172), and Henderson (173) went on to study the demethoxylation of model aromatic compounds, using mycelial mats and germinated spore suspensions of *Haplographium, Hormodendron, and Penicillium.* Low concen-

trations of substrate had to be used to avoid inhibitions. The detailed results varied somewhat with the species but, in general, all three monomethoxybenzoic acids were oxidized. The corresponding hydroxybenzoic acids were detected after incubation, but the fate of the methoxyl group could not be ascertained. The three hydroxybenzoic acids were oxidized and in the case of the *para* derivative, protocatechuic acid was detected at the end. With both sets of substrates the *para* derivatives were usually attacked most rapidly, and this preferential attack on the *para* position was further exemplified in the metabolism of veratric acid (3:4-dimethoxybenzoic acid). This gave rise to vanillic acid which later disappeared from the medium. The 3:4- and 2:4-dimethoxy derivatives and the 2:3:4-trimethoxy derivatives were attacked less rapidly than the monomethoxy derivatives.

The discovery that the selective herbicide 2:4-dichlorophenoxyacetic acid (2:4-D; XXIX) is broken down in soil (174) has been followed by the isolation of various organisms capable of using this compound and 4-chloro-2-methylphenoxyacetic acid (MCPA; XXXII) as carbon source (175 to 178). Researches into the metabolism of these two substances have recently been undertaken.

Rogoff & Reid (178) have used a *Corynebacterium* which grows on a synthetic medium containing as much as 0.3 per cent of 2:4-D. Spectrophotometric assays showed a virtually complete disappearance of 2:4-D from growing cultures. No spectroscopically detectable products were formed and the chlorine of the substrates was converted to inorganic chloride. When 2:4-D was oxidized by washed cells almost the whole of the substrate carbon was recovered as carbon dioxide. The molecule is thus completely destroyed.

Audus & Symonds (177) obtained evidence that two phytotoxic intermediates are formed during the breakdown of 2:4-D but did not identify them; evidence of other workers indicates that these intermediates may be 2:4-dichlorophenol (XXX) and 4-chlorocatechol (XXXI). Steenson & Walker (179, 180) showed that the oxidation of 2:4-D by *Flavobacterium peregrinum* and an *Achromobacter* species is adaptive. In both cases, age of the culture was an important factor in obtaining active washed cell suspensions. Adapted cells of these species oxidized 2:4-dichlorophenol and 4-chlorocatechol without lag and Rogoff & Reid (178) also noted that their organism oxidized 2:4-dichlorophenol. The probable route is as shown in Figure 6. Similar evidence indicates that MCPA is oxidized via 5-chloro-2-cresol (XXXIII).

The nature of the process whereby the halogen is removed from the molecule is unknown and little is known about the stage at which this occurs beyond the fact that it does not initiate the attack upon the molecule [cf. the metabolism of mono-halogenated naphthalenes which give rise to the corresponding derivatives of salicylic acid (181)]. As with the cresols and methoxylated derivatives, the first step appears to be the removal of the side

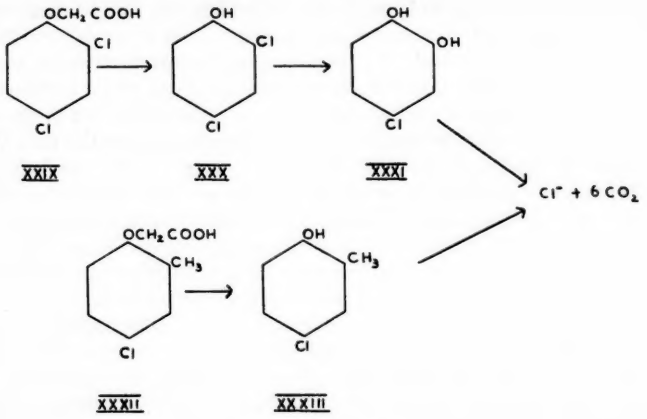



FIG. 6

chain and specificity studies indicate that substitution in the *para* position is again a prerequisite for enzymic attack. A preliminary report by Evans & Smith (176), however, indicates that in the breakdown of 4-chlorophenoxyacetic acid, hydroxylation of the nucleus in position 2 precedes removal of the side chain. It would seem, therefore, that different organisms may utilize different pathways for the oxidation of chlorophenoxy derivatives.

Much of the recent work on the oxidation of these more complex aromatics has been largely confined to the isolation of compounds from growing cultures and to tests for the oxidation of suspected intermediates by washed cells. The full requirements for use of the sequential induction argument have not always been met; authors have frequently failed to establish that the suspected intermediates are not oxidized by cells growing on nonaromatic substrates. In many cases, the products of oxidation have not been identified, much less estimated, and few reactions have been demonstrated in cell-free extracts. Interpretation of data is frequently hampered by the fact that many of the suspected intermediates are inhibitory at concentrations commonly employed in manometric experiments. Some of the metabolic routes given here must therefore be regarded at present as convenient working hypotheses rather than proven pathways.

### Tricarboxylic Acid Cycles and the Synthesis of 4-Carbon Dicarboxylic Acids

Krebs, Gurin & Eggleston (182) suggested that, in growing cells, the reactions of the tricarboxylic acid cycle may serve, not merely to provide energy by the oxidation of acetate, but also to supply carbon skeletons for amino acid synthesis. Tracer studies with *Escherichia coli, Torulopsis utilis, Rhodospirillum rubrum, Neurospora crassa,* and *Zygorrhyncus moelleri*

support this hypothesis (183 to 188). The tapping off of intermediates in this manner demands the *de novo* synthesis of 4-carbon dicarboxylic acids by the cell if the activity of the cycle is to be maintained. This process would be necessary on a small scale in any case for the duplication of the catalytic machinery which is a necessary consequence of cell division. The formation of 4-carbon acids is thus an integral part of cell synthesis but, in addition, many bacteria and molds produce substantial amounts of tricarboxylic acid cycle intermediates in the medium as catabolic end products.

The synthesis of 4-carbon acids from 3-carbon substrates and glucose can be accounted for by known mechanisms. Thus, malate may be produced from pyruvate by the "malic enzyme" (189, 190), and oxalacetate may be produced by the carboxylation of phosphoenolpyruvate, a reaction known to occur in animal tissues (191). In the case of *Propionibacter* and *Veillonella* there is conclusive evidence that the carboxylation of pyruvate is involved in the formation of succinate (192 to 195). Tracer studies with growing cultures of *Clostridium kluyveri* and *Escherichia coli* indicate that in these organisms the condensation of carbon dioxide with a 3-carbon intermediate is involved in amino acid synthesis (196, 197).

The synthesis of 4-carbon acids from 2-carbon substrates, particularly acetate, has remained relatively obscure. Although 4-carbon acids might be produced by the carboxylation of acetate to yield pyruvate which could then undergo a further carboxylation, the net synthesis of pyruvate from 1-carbon substrates plus acetate has never been satisfactorily demonstrated in a cell-free extract, despite the fact that the exchange of carbon dioxide and formate with the carboxyl of pyruvate is well-established (198 to 201). An alternative suggestion was made by Thunberg (202), namely, that succinate might be formed by the oxidative "tail-to-tail" condensation of two molecules of acetate.

$$2\ CH_3COOH \rightarrow COOH \cdot CH_2 \cdot CH_2 \cdot COOH + 2(H) \qquad 14.$$

This hypothesis has received some support from isotopic tracer studies. For example, the work of Foster and his co-workers (203, 204) with *Rhizopus nigricans*, growing on ethanol, indicates that the methene and carboxyl groups of the fumarate produced in the medium are derived from the methyl and carbinol groups of the ethanol, respectively. The last few years have seen the demonstration in cell-free extracts of a number of reactions which may be concerned in such a synthesis of 4-carbon acids from 2-carbon substrates. At present, the reports available are mainly of a preliminary nature and few experimental data have been presented. However, in view of the advances made, comment at this stage seems worthwhile.

Barron & Ghiretti (205) observed the reduction of methylene blue by cell-free extracts of *Corynebacterium creatinovorans* in the presence of acetate. The same effect was also obtained with extracts of Baker's yeast, *Pseudomonas fluorescens, Aspergillus, Tetrahymena geleii,* preparations

from animal tissues, and with dialyzed extracts of *Aerobacter aerogenes* supplemented with CoA, DPN, FAD, and $Mg^{++}$.

Seaman & Nashke (206) studied in detail the cleavage of succinate by extracts of *Tetrahymena* and found that in addition to CoA, their system required ATP. They formulated the reaction as follows:

$$\text{Succinate} + 2\ \text{ATP} + 2\text{CoA} + \text{DPNH} + \text{H}^+ \rightarrow 2\ \text{Acetyl CoA} + 2\text{ADP} + \text{DPN}^+ + 2\ \text{P}_i \quad 15.$$

The formation of acetyl CoA was demonstrated, using a hydroxylamine trap and with lactate-lactic dehydrogenase as H-donor. The formation of succinate by the reverse reaction was also demonstrated, using acetyl-P plus phosphotransacetylase as the acetyl donor system. The observed reactions were very slow and the amounts of products formed were low in comparison with the amounts of substrates added. In addition, the possibility that the acetyl CoA might have arisen from pyruvate formed by the H-donor system was not excluded.

In a later paper (207), this latter point has been cleared up by experiments in which the lactate-lactic dehydrogenase was replaced by formate-formic dehydrogenase as the H-donor. Furthermore, in experiments with labelled succinate, all the succinate disappearing was accounted for by the acetate produced, and the methyl and carboxyl groups of acetate were derived from the methylene and carboxyl groups, respectively, of succinate. Both the reduction of succinate by reduced DPN and the reverse reaction were demonstrated spectrophotometrically and the equilibrium constant determined. The enzyme preparation used in these later studies was purified 150-fold but in view of the complexity of the reaction it is difficult to believe that only a single enzyme is involved.

A second system bringing about a synthesis of a 4-carbon acid is the "malate synthetase" of Wong & Ajl (208). These workers used a fiftyfold purified enzyme from acetate-grown *E. coli* to demonstrate the synthesis of malate from glyoxylate plus acetyl-P, in the presence of added phosphotransacetylase. The system required CoA, $Mg^{++}$, and cysteine and the conversion of approximately half of the substrates to malate was observed. The preparation was free of fumarase, Ochoa's condensing enzyme, isocitratase, and glyoxylate reductase and it was subsequently shown that acetyl CoA could replace the acetyl-P plus phosphotransacetylase (209). The reaction (Equation 16) is thus analogous to the formation of citrate by Ochoa's condensing enzyme (210).

$$\text{COOH}\cdot\text{CHO} + \text{CH}_3\cdot\text{COS CoA} \rightarrow \text{COOH}\cdot\text{CHOH}\cdot\text{CH}_2\cdot\text{COOH} + \text{CoASH} \quad 16.$$

A third reaction of interest has been reported by Krakow & Barkulis (211). With extracts obtained from glycollate-grown cells of *E. coli*, a disappearance of glyoxylate was observed under anaerobic conditions and this disappearance was accompanied by the release of one mole of carbon

dioxide for every two moles of glyoxylate added. After fractionation of the crude extract, $Mg^{++}$ and diphosphothiamine stimulated the reaction. An end product was detected whose behavior on chromatograms suggested that it was 3-hydroxypyruvic acid or its isomer, tartronic semialdehyde. The authors suggested that this product is formed by the combination of a 1-carbon unit derived from the aldehyde group of one glyoxylate molecule, with a second molecule thus:

$$COOH \cdot CHO + CHO \cdot COOH \rightarrow CO_2 + CH_2OH \cdot CO \cdot COOH \qquad 17.$$

Although this reaction does not produce a 4-carbon acid directly the hydroxypyruvate might well give rise to pyruvate in the intact cell whence 4-carbon acid synthesis via the malic enzyme would appear possible. If this latter step utilized the carbon dioxide produced from glyoxylate, then the carbon atoms of the malate formed would be derived from the same glyoxylate carbons as in a tail-to-tail combination of 2-carbon units.

Thus, at least three enzymic processes are known which might play a part in the synthesis of 4-carbon dicarboxylic acids from 2-carbon substrates and the question of their relative importance arises. It would seem unlikely that the system described by Seaman plays a significant role in succinate synthesis in view of the low rates observed. With DPN as H-acceptor and acetyl-P as acetyl-donor, the equilibrium does not favor succinate synthesis, but it is possible that conditions might be more favorable for synthesis in the intact cell if the reaction were coupled with suitable exergonic reactions. If either malate synthetase or the system of Krakow & Barkulis are important in 4-carbon acid synthesis, then it would appear at first sight that glyoxylate would be required in substrate amounts. This presents a difficulty in the case of organisms growing on acetate since no enzyme system has yet been discovered which will oxidize acetate to glycollate directly, although enzymes are known which oxidize the latter to glyoxylate (212, 213). However, this difficulty has been resolved by the recent work of Kornberg and his collaborators which has led to a new concept concerning the manner in which malate synthetase is fitted into the metabolic machinery of the cell.

Kornberg & Madsen (214, 215), working with acetate-grown cells of *Pseudomonas fluorescens* KB1 (216), have demonstrated that extracts of this organism contain aceto-CoA-kinase (217), malate synthetase (208, 209), and isocitratase (218 to 221). The data so far presented show only a small net conversion of acetate plus glyoxylate to malate by crude extracts, but when glyoxylate was replaced by isocitrate, almost all of the latter was utilized and malate and succinate appeared in approximately equivalent amounts. This indicates that the reactions of the acetate-activating enzyme (Equation 18a), isocitratase (Equation 18b), and malate synthetase (Equation 18c) can be coupled together to give the overall reaction indicated by Equation 18d.

$$\text{ATP} + \text{CoA} + \text{Acetate} \longrightarrow \text{Acetyl CoA} + \text{AMP} + \text{P-P} \qquad 18a.$$
$$\text{Isocitrate} \longrightarrow \text{Glyoxylate} + \text{succinate} \qquad 18b.$$
$$\text{Glyoxylate} + \text{acetyl CoA} \longrightarrow \text{Malate} + \text{CoA} \qquad 18c.$$

$$\text{ATP} + \text{Isocitrate} + \text{Acetate} \rightarrow \text{Malate} + \text{Succinate} + \text{AMP} + \text{P-P} \qquad 18d.$$

Kornberg & Madsen (214) also showed that their extracts contained Ochoa's condensing enzyme and further, that washed cells oxidized all of the intermediates of the tricarboxylic acid cycle. The organism thus contains all of the enzymes of the cycle when grown on acetate. The hypothesis has therefore been put forward by Kornberg & Madsen (214) and developed in detail by Kornberg & Krebs (222) [cf. Wong & Aji (209)], that the composite reaction (18d) may replace those steps of the conventional tricarboxylic acid cycle leading from isocitrate to malate. In conjunction with the remaining steps of the conventional cycle (summarized by Equation 18e) this gives a modified cycle, the overall result of which is expressed by Equation 18f. Each turn of this new cycle accomplishes the net synthesis of one molecule of succinate by the oxidation of two molecules of acetate.

$$\text{ATP} + \text{Malate} + \text{Acetate} \rightarrow \text{Isocitrate} + 2(\text{H}) + \text{AMP} + \text{P-P} \qquad 18e.$$
$$2\,\text{ATP} + 2\,\text{Acetate} \longrightarrow \text{Succinate} + 2(\text{H}) + 2\,\text{AMP} + 2\,\text{P-P}$$
$$18f\ (= 18d + 18e).$$

Although the mechanism is complex, the overall reaction is formally identical with that postulated by Thunberg, except for the participation of ATP. Furthermore, when the fate of the individual acetate carbons in the modified cycle is considered, it is found that the carboxyl and methyl carbons of acetate give rise to the carboxyl and methylene carbons, respectively, of succinate, as in the Thunberg reaction. Since glyoxylate plays a catalytic role in this new cycle and is the one intermediate not shared with the conventional tricarboxylic acid cycle, it was proposed that this new reaction sequence be known as the "glyoxylate cycle."

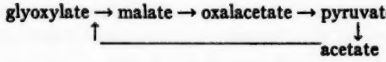
The evidence that the formation of 4-carbon acids during growth does indeed occur by way of the glyoxylate cycle is strongest in the case of *Pseudomonas*. In addition to the demonstration that the necessary enzymes are present in *Pseudomonas* KB1, Kornberg & Madsen (214) state that in short-term experiments malate was the only labelled product appearing when glyoxylate and $^{14}$C-labelled acetate were incubated with cell-free extracts. It is also stated (214, 222, 223, 224), that data obtained with growing cultures indicate that acetate enters the tricarboxylic acid cycle at two points to give malate at one and citrate at the other. This clearly disposes of the possibility that there is a direct condensation of two acetate molecules to yield succinate in this organism and establishes beyond reasonable doubt the *in vivo* importance of the glyoxylate cycle in *Pseudomonas*. The full reports of these investigations will be awaited with considerable interest.

There is also good evidence for the occurrence of the new cycle in the *Vibrio* O1 of Happold & Key (156). Dagley & Patel (225) found that after growth on acetate or fatty acids, washed cells of this organism accumulate 2-ketogluturate during the oxidation of acetate. Callely, Dagley & Hodgson (226) have demonstrated the presence of isocitratase and malate synthetase in cell-free extracts of this organism. They also observed that at low concentrations acetate was not oxidized unless fumarate was added as "sparker;" at higher concentrations, acetate was oxidized at an appreciable rate when added alone. Under these latter conditions the rate of oxidation was increased on addition of glyoxylate although glyoxylate itself was not oxidized when added alone. This observation is of interest as the stimulation of acetate oxidation by glyoxylate would be expected, given suitable experimental conditions, as a consequence of the Kornberg-Madsen-Krebs hypothesis. After growth on succinate, or aromatic compounds known to give rise to succinate, the organism did not accumulate 2-ketoglutarate when oxidizing acetate (225); such cells contained only one-tenth of the isocitratase activity present in cells grown on acetate or octanoate (226).

With regard to molds, Kornberg & Krebs (222) point out that the quantitative isotope data on the fumarate formed by *Rhizopus nigricans* (203, 204) can be better accounted for in terms of the glyoxylate cycle than in terms of a dicarboxylic acid cycle. Isocitratase has already been shown in *Rhizopus* by Olson (219).

The distribution of isocitratase and malate synthetase, the two characteristic enzymes of the glyoxylate cycle has been studied to some extent and the results so far obtained lend support to the postulated role of the cycle in 4-carbon-acid synthesis. In addition to the instances already mentioned, isocitratase has been demonstrated in *Penicillium chrysogenum, Aspergillus niger,* baker's yeast, *Pseudomonas aeruginosa, Pseudomonas putrefaciens, Azotobacter, Escherichia coli, Serratia marcescens, Acetobacter aceti,* and *Micrococcus lysodeikticus* (218, 219, 221, 227, 228, 229). Smith & Gunsalus (227) found that the enzyme is apparently formed adaptively when growth conditions require the aerobic synthesis of dicarboxylic acids from acetate. Thus, *Acetobacter aceti, E. coli* and *P. aeruginosa* all contained isocitratase after aerobic growth on acetate, but when acetate was replaced by glucose or glycerol, the enzyme was absent from the first two organisms and had reduced activity in the last. Less is known as yet about the distribution of malate synthetase. As far as we are aware, the only organisms in which it has been demonstrated to date are *Escherichia coli, Aerobacter aerogenes, Corynebacterium creatinovorans, Pseudomonas fluorescens* and *Vibrio* O1 (208, 209, 214, 226). Wong & Ajl (209) reported that with the first four of these organisms, malate synthetase was found only after growth on acetate. Nevertheless, it is not yet possible to assess how widespread this modified cycle is. Both isocitratase and malate synthetase may have functions other than their part in the glyoxylate cycle. Thus, isocitratase may serve to produce glyoxylate for glycine synthesis as suggested by Campbell, Smith

& Eagles (218), and malate synthetase may be involved in some form of dicarboxylic acid cycle. For example, the oxidation of glyoxylate produced from glycine or purines (230) might well occur by the following reaction sequence:




19.

Although the glyoxylate cycle can account for 4-carbon acid synthesis from ethanol and acetate, there remain several unsolved problems with regard to the metabolism of 2-carbon acids. Tracer studies with *Clostridium kluyveri* (196) and *E. coli* (183, 197) suggest that other pathways must exist for the assimilation of acetate. In addition, the utilization of glycollate and glycine for growth is not understood at present. For example, the isotope data of Jayasuriya (231, 232) on the synthesis of citrate from glycollate by an oxalate-decomposing *Pseudomonas* cannot be explained merely in terms of the citrate and glyoxylate cycles. The stimulation of autorespiration by glycollate in *Prototheca zopfii* is a further puzzling phenomenon since glycollate is without effect on acetate oxidation in this organism (233). Nevertheless, the present evidence indicates that the glyoxylate cycle is of considerable importance in strict aerobes growing on acetate and this new concept undoubtedly opens up new lines of approach to the problems of 2-carbon acid metabolism.

What may be part of a highly specialized pathway for the formation of 4-carbon acids from pentoses has been discovered by Weimberg & Doudoroff (234). Cell-free extracts obtained from *Pseudomonas saccharophila* grown on L-arabinose were found to oxidize this substrate to 2-ketoglutarate which could presumably give rise to 4-carbon acids.

The earlier investigations of Krampitz's group on the occurrence of the tricarboxylic acid cycle in *E. coli* and *Micrococcus lysodeikticus* (235, 236, 237) have been extended to baker's yeast by DeMoss & Swim (238). Using nonproliferating cells, they obtained isotope data which indicate that the tricarboxylic acid cycle is the major pathway of acetate oxidation in baker's yeast under these conditions. Eaton & Klein (239) have studied the oxidation of glucose by resting cell suspensions prepared from pure cultures of *Saccharomyces cerevisiae* and have obtained evidence that glucose first gives rise to ethanol by the Embden-Meyerhof pathway. This is then converted to acetate which accumulates before being oxidized via the tricarboxylic acid cycle. The oxidation of the accumulated acetate appears to be adaptive. No evidence was found in support of the view that a hexomonophosphate pathway of the type suggested by Horecker & Mehler (240) occurs in the strain used by Eaton & Klein.

Fisher (241) has reported some interesting findings on the role of the tricarboxylic acid cycle in the conjugation of *E. coli* K 12, following the observation made by Hayes (242) that glucose and aspartate are required

for the formation of zygotes when donor and recipient strains are incubated together. Fisher found that under suitable conditions, glucose could be replaced by acetate, and aspartate by glutamate, succinate, fumarate or malate. Zygote formation was inhibited by malonate, monofluoroacetate, and cyanide and was very much reduced when $N_2$ replaced air as the gas phase. These observations indicate that under the conditions used, the formation of zygotes requires the operation of the tricarboxylic acid cycle. DNP was also inhibitory, suggesting that the role of the cycle is to provide energy rather than intermediates. Further experiments (243) showed that the number of zygotes formed was drastically reduced when the donor strain was starved, whereas starving the recipient strain had no effect. It was concluded that only the donor cells require energy and evidence was obtained that the endergonic processes involved are the establishment of effective contact with the recipient cell and the transfer of genetic material.

### Miscellaneous

*Itaconate.*—Three papers have been published by Bentley & Thiessen (244, 245, 246) on the mechanism of itaconate formation by *Aspergillus terreus*. Bentley & Thiessen (244) examined the labelling of itaconate when the mold was fed with either acetate-1-$^{14}$C, succinate-2-$^{14}$C, succinate-1-$^{14}$C, or glucose-1-$^{14}$C. The carboxyl of acetate-1-$^{14}$C appeared in $C_1$ and $C_4$ of itaconate (the numbering systems adopted for itaconate and related acids are given in Figure 7a). The succinate carboxyl also appeared in itaconate $C_1$ and $C_4$ and the methylene carbons in $C_2$ and $C_3$. Thus, it would seem that the succinate carbon skeleton is converted unchanged into itaconate. The behavior of acetate can be explained as follows: when acetate is converted to succinate by one turn of the tricarboxylic acid cycle acetate $C_1$ becomes a succinate carboxyl and acetate $C_2$ becomes a methylene carbon. And hence, where succinate goes, there also will go acetate. Extracts of the mold were prepared which would decarboxylate *cis*-aconitate (246). These extracts also contained aconitase and, in consequence, both *d*-isocitrate and citrate were converted to itaconate with the formation of $CO_2$. The aconitase was inhibited by both *l*-isocitrate and fluorocitrate. These observations provide an explanation for the conversion of succinate and acetate to itaconate and are shown in Figure 7b (for convenience preliminary conversion of succinate to oxalacetate via the tricarboxylic acid cycle is assumed). While in theory two forms of *cis*-aconitate should be produced (Fig. 7b), only one form is made by the aconitase from animal tissues owing to three point attachment (247); the alternative configuration of *cis*-aconitate is indicated in Figure 7b by broken arrows. If the mold aconitase acts in a similar fashion it is clear that the decarboxylation of *cis*-aconitate must involve not only removal of $CO_2$ but also the migration of the double bond. The only other way in which the isotope distribution results could be explained involves the assumption that the mold aconitase behaves differently from the enzyme of animal tissues. Bentley & Thiessen (246) tested these

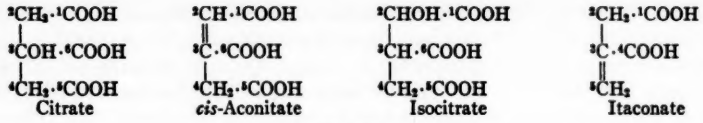



FIG. 7a

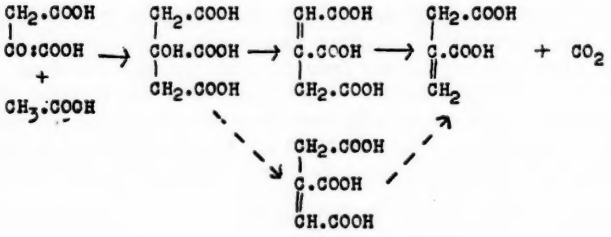


FIG. 7b

two possibilities using citrate-1 : 6-$^{14}$C and isocitrate-5 : 6-$^{14}$C; this latter compound was made from glyoxylate and carboxyl-labelled succinate by the action of isocitratase. Citrate-1 : 6-$^{14}$C gave itaconate containing all the isotope added, and the $CO_2$ was unlabelled. Isocitrate 5 : 6-$^{14}$C on the other hand gave labelled $CO_2$ and labelled itaconate. These observations show very clearly that it is a primary carboxyl group which is removed and that decarboxylation is accompanied by a shift of the double bond; they also show that the mold aconitase resembles the animal tissue enzyme in its action.

*Metabolism of polyhydric alcohols.*—Earlier work (248) has indicated that strains of *Aerobacter aerogenes* metabolize glycerol by two different routes. In strain 1033 dihydroxyacetone is the intermediate whereas L-glycerol-1-phosphate is the intermediate in strain 1041. There is evidence (249) that rat liver distinguishes between the two enantiomorphs of glycerol-1-$^{14}$C, for D-glycerol-1-$^{14}$C is converted to glycerol-1-$^{14}$C-1-phosphate, and L-glycerol-1-$^{14}$C to glycerol-1-$^{14}$C-3-phosphate. Rush *et al.* (130) have used the enantiomorphs of labelled glycerol-1-$^{14}$C to study the metabolism of glycerol by their two strains of *A. aerogenes*. The organisms were allowed to oxidize D-, L-, and DL-glycerol-1-$^{14}$C in the presence of arsenite and the pyruvate which accumulated was isolated and degraded.

The pyruvate formed by strain 1033 from D-, L-, and DL-glycerol-1-$^{14}$C in each case contained 50 per cent of the total counts in the carboxyl group and this is to be expected if dihydroxyacetone is an intermediate. On the other hand, strain 1041 converted the D-form to pyruvate with 4.5 per cent total counts in the carboxyl, the L-form to pyruvate with 96.9 per cent total counts in the carboxyl, and the DL-form to pyruvate containing 55.5 per cent total counts in the carboxyl. These results are to be expected if strain 1041 con-

verts D-glycerol-1-$^{14}$C to glycerol-1-$^{14}$C-1-phosphate and L-glycerol-1-$^{14}$C to glycerol-1-$^{14}$C-3-phosphate.

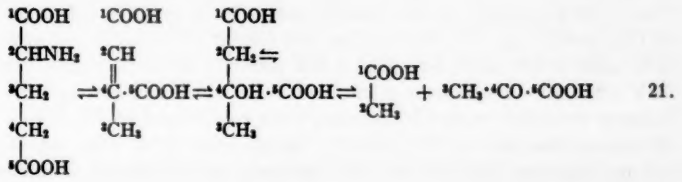
Strain 1041, but not strain 1033, contained glycerokinase and only strain 1033 possessed the glycerol dehydrogenase. These experiments show conclusively that strain 1033 oxidizes glycerol via dihydroxyacetone whereas in strain 1041 L-glycerol-1-phosphate is the intermediate.

Two soluble dehydrogenases oxidizing sorbitol have been found in *Acetobacter suboxydans* (250, 251). One, which requires TPN, oxidizes sorbitol to sorbose. The other is specific for DPN and oxidizes sorbitol to fructose.

α-*Acetolactate.*—Umbarger, Brown & Eyring (252), using a valineless mutant of *E. coli,* have found that α-acetolactate accumulates when the mutant is grown on minimal medium supplemented with valine. Cell-free extracts of this mutant produced some ten times as much α-acetolactate as did comparable extracts prepared from the wild type. The wild type converted α-acetolactate-β-$^{14}$C to L-valine and L-leucine, whereas a suitably blocked mutant formed α:β-dihydroxyisovaleric acid. Willson & Adelberg (253) have examined the compounds produced by a valineless mutant of *Neurospora* and have isolated 4-hydroxy-4-methyl-2-ketoglutarate and citramalate. If α-acetolactate is an intermediate in the synthesis of valine and leucine, as would appear from the evidence of Umbarger *et al.* (252), we have here another example of a single metabolic process performing two quite distinct functions, for, as was shown by Juni (254), α-acetolactate is the precursor of acetoin and 2:3-butanediol, both major end products of the *Aerobacter* fermentation. The specificity of the 2:3-butanediol dehydrogenases of various organisms has been described by Juni & Heym (255). The enzymes, which require DPN, are relatively unspecific. α-Acetolactate may also be a precusor of pantothenic acid since McIntosh, Purko & Wood (256) have extracted from *E. coli* an enzyme which converts 2-ketoisovalerate to 2-ketopantoate by the addition of formaldehyde. The enzyme does not require folic acid and is activated by either $Co^{++}$, $Mn^{++}$, or $Mg^{++}$.

$$(CH_3)_2\cdot CH\cdot CO\cdot COOH + HCHO \rightarrow CH_2OH\cdot C\cdot(CH_3)_2\cdot C{:}O\cdot COOH \qquad 20.$$

*Citramalate and mesaconate.*—Citramalate has come to the fore as an intermediate in the fermentation of glutamate by *Clostridium tetanomorphum* (10) in which butyrate, acetate, hydrogen, carbon dioxide, and ammonia are the products (257). Extracts of this organism convert glutamate to acetate and pyruvate with mesaconate and (+) citramalate as intermediates.




21.

Each of the reactions is reversible and in each case the equilibrium is to the right. Citramalase, which catalyses the hydration of mesaconate, is an enzyme of the same general type as fumarase, aconitase, and crotonase. It resembles aconitase from animal tissues in that it requires both $Fe^{++}$ and cysteine as cofactors [Morrison (258)]. The initial step, the conversion of glutamate to mesaconate is very complex, involving as it does both a deamination and a rearrangement of the $C_3$, $C_4$, and $C_5$ of glutamate, and the detailed mechanism of this reaction must still be worked out. The cleavage of citramalate resembles the cleavage of citrate to oxalacetate and acetate by citratase in so far as free acetate is produced rather than acetyl CoA. This means that the sole energy-producing reaction at the substrate level is the breakdown of pyruvate, presumably to acetyl CoA. If all of the acetyl CoA so formed is used to synthesize ATP then the yield of ATP is 1 mole/mole glutamate fermented. However, butyrate is one of the end products and this, presumably, is formed by the overall reaction.

$$\text{Acetate} + \text{Acetyl CoA} + 4(H) \rightarrow \text{Butyrate} + \text{CoA} \qquad 22.$$

This means that the ATP yield will be less than unity by an amount proportional to the butyrate formed. On the other hand, the possibility exists here, as Barker has suggested for *Clostridium kluyveri* (10) that additional ATP may be synthesized by the coupling of phosphorylation to the electron transport system (see p. 152). This work of Barker and his colleagues, has brought two new intermediates, (+) citramalate and mesaconate, to the attention of those working on intermediary metabolism and it may well prove to be as far reaching in its effects as their previous work on *C. kluyveri.*

*Formate.*—Peck & Gest (259) have made further progress in their analysis of the formic hydrogenlyase system. Extracts of two organisms, an anaerogenic strain of *E. coli* (WR2) and an atypical *Klebsiella* (WR3), neither of which contained formic hydrogenlyase, were assayed for hydrogenase and formic dehydrogenase activity. The hydrogenase assay was based on the reduction of either benzyl viologen or methylene blue by molecular hydrogen, and only extracts of WR2 contained significant amounts of this enzyme. Formic dehydrogenase was found in WR3 but not in WR2. When extracts of WR2 and WR3 were combined, the mixture had powerful formic hydrogenlyase activity [cf. Gest & Peck (260)]. In the presence of excess WR2 extract the rate of hydrogen formation from formate by the mixture was proportional to the formic dehydrogenase activity of WR3 and this reaction was used to assay formic dehydrogenase activity. Two other methods were also used to assay this enzyme, based on the rate of $CO_2$ production when the enzyme and formate were incubated anaerobically with either methylene blue (MB activity) or with benzyl viologen (BV activity). When crude extracts of WR3 which had been given a preliminary treatment with ribonuclease, were centrifuged at 120,000 *G*, only the supernatant showed BV activity. On the other hand, both supernatant and particles had MB activity. The chelating agent, catechol disulphonate,

inhibited MB activity, but not BV activity. It was also possible to separate the two activities with ammonium sulphate. These results can be explained in two ways: either there are two distinct formic dehydrogenases, one able to reduce methylene blue and the other benzyl viologen or else there is one enzyme and two different carriers, one which transfers hydrogen to methylene blue, the other to benzyl viologen. Peck & Gest call the latter carrier $X_1$. Only benzyl viologen preparations could be coupled with the hydrogenase preparation to produce hydrogen from formate, suggesting that they contain the formate-activating component of the formic hydrogenlyase system, and in agreement with this it was found that benzyl viologen activity, like formic hydrogenlyase, was only present when the organisms were grown anaerobically.

The hydrogenase system of WR2 was also examined and this likewise could be separated into two components. One was soluble and reduced both methylene blue and benzyl viologen with hydrogen. The second was insoluble; on mixing it with formic dehydrogenase (BV) hydrogen was produced from formate. The insoluble component could be replaced by methyl viologen in the hydrogen evolution test, and Peck & Gest refer to the active component as carrier $X_2$.

The formic hydrogenlyase system would thus appear to consist of 4 components, viz., formic dehydrogenase, carrier $X_1$, carrier $X_2$, and hydrogenase, with the possibility that formic dehydrogenase and $X_1$ are identical and specific for the formic hydrogenlyase system. It would be of great interest to know whether this mixture of enzymes and carriers synthesizes formate from hydrogen and carbon dioxide in the same way as whole cells, [Woods (261)]. Peck & Gest (262) have purified the hydrogenase of *Clostridium butylicum* about 200 times. The final preparation which had a slightly yellow tinge, was partially inactivated by dialysis in the presence of *o*-phenanthroline; the activity was restored by addition of $Fe^{++}$. When preparations which had been dialyzed in this way, were aged for a few days, in addition to $Fe^{++}$, FAD was also required for full activity. The authors conclude that this hydrogenase is a metallo-flavoprotein. It reduced benzyl viologen but not methylene blue.

An enzyme oxidizing formate has been prepared from *Aspergillus niger* by Hauge (263). Mycelia grown in glucose did not contain the enzyme but if such mycelia were incubated with formate prior to extraction, then the extracts contained the enzyme. No cofactors were required and the enzyme did not react with methylene blue. The amount of oxygen consumed exceeded 0.5 $O_2$/mole formate and some evidence was obtained for the presence of hydrogen peroxide in the reaction mixture at the end of the experiment. The author suggests that this enzyme catalyses the following reaction:

$$HCOOH + O_2 \rightarrow H_2O_2 + CO_2 \qquad 23.$$

Hug & Sagers (264) have purified a formic dehydrogenase from *Clostridium acidiurici* which reacts quantitatively with benzyl viologen.

*Lactic acid.*—The lactic oxidative decarboxylase of *Mycobacterium phlei* has been crystallized by Sutton (265). The enzyme catalyzes the following reaction:

$$CH_3CHOH\ COOH + O_2 \rightarrow CH_3COOH + CO_2 + H_2O \qquad 24.$$

Flavin mononucleotide is the prosthetic group of the enzyme. The mechanism of this reaction has been investigated by Sutton & Hayaishi using $^{18}O_2$ and $H_2{}^{18}O$ (266). Their results, although curious, were quite clear cut and are shown by the following reactions:

$$CH_3CHOH\ COOH + {}^{18}O_2 \rightarrow CH_3C^{18}OOH + CO_2 \qquad 25a.$$
$$CH_3CHOH\ COOH + O_2 + H_2{}^{18}O \rightarrow CH_3COOH + C^{18}O_2 \qquad 25b.$$

*Oxalate metabolism.*—Enzymes concerned with oxalate metabolism have been studied by Hayaishi and his colleagues. Extracts have been prepared from *Aspergillus niger* which bring about a hydrolytic cleavage of oxalacetate to oxalate and free acetate (267). The enzyme, which is highly specific, appears to require only $Mn^{++}$ for full activity.

A new enzyme, which converts oxalate to formate and carbon dioxide has been extracted from the wood-destroying fungus *Collyvia veltipes* by Shimazono & Hayaishi (268) and purified some 400-fold. While oxygen does not appear to be involved, it was found that the enzyme required the presence of a small amount of oxygen for activity. It is therefore possible that the reaction proceeds in two steps:

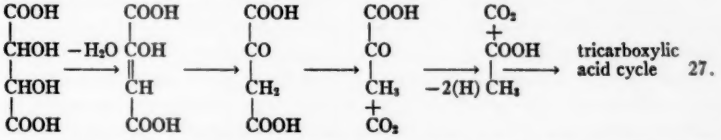
$$\begin{matrix} COOH \\ | \\ COOH \end{matrix} + O_2 \longrightarrow 2\ CO_2 + H_2O_2 \qquad 26a.$$

$$\begin{matrix} COOH \\ | \\ COOH \end{matrix} + H_2O_2 \rightarrow 2\ HCOOH + O_2 \qquad 26b.$$

However, $H_2O_2$ had no effect on the reaction and addition of catalase did not inhibit it. The enzyme differs from the bacterial enzyme studied by Jakoby, Ohmura & Hayaishi (269) which requires ATP, CoA, diphosphothiamines, $Mg^{++}$, and acetate.

*Tartrate.*—Studies on the breakdown of tartrate by a variety of microorganisms have been reported from several laboratories in recent years. The results obtained confirm the suggestion put forward by Barker (270) as a result of work with *Aerobacter aerogenes,* that oxalacetate and pyruvate are intermediates. Cell suspensions and cell-free extracts have been obtained from *Pseudomonas incognita, P. putida, P. fluorescens,* and a Gram-positive bacillus (271 to 274), which produce carbon dioxide from both tartrate and oxalacetate. The primary reaction appears to be a dehydration to give oxalacetate [cf. the dehydration of 6-phospho-gluconate, galactonic acid and D-arabonic acid (12, 32, 33)], but in order to demonstrate the formation of this intermediate from tartrate the authors have had to resort to the use of trapping agents and inhibitors of oxalacetate break-

down. Even so, the amounts of oxalacetate formed have usually been small or have not been recorded. The only satisfactory quantitative data on the dehydration is contained in the paper by Shilo (*273*), who used ethylenediaminetetracetic acid (EDTA) to inhibit the breakdown of oxalacetate, the appearance of which was followed by spectrophotometry. With extracts of *P. fluorescens,* he observed the conversion of 80 per cent of added tartrate to oxalacetate. The accumulated product disappeared completely upon adding $Mg^{++}$ to relieve the inhibition of oxalacetic decarboxylase by the EDTA. In the absence of ethylenediaminetetracetic acid a 90 per cent conversion of tartrate to pyruvate was demonstrated. The other authors all showed the formation of acetate or active acetate as an end product and la Rivière (*272*) found that extracts of *Pseudomonas putida* decarboxylated pyruvate. All authors observed, under suitable conditions, the production of more than one mole of carbon dioxide per mole of tartrate metabolized. The breakdown of tartrate appears to occur by the following pathway in these organisms.




27.

Results obtained with isotopic $CO_2$ (*271*) and with inhibitors (*271, 272*) are in keeping with this scheme.

The above experiments have, for the most part, been carried out under anaerobic conditions. In the fermentation of tartrate by growing cultures of *Clostridium butyricum* (*275*) and of the organism studied by Krampitz & Lynen (*274*) the oxidation of pyruvate in the above sequence is balanced by the evolution of molecular hydrogen or the formation of butyrate or lactate. In the studies of Nomura (*271*) and of la Rivière (*272*) which were carried out with *Pseudomonas* preparations under anaerobic conditions, oxalacetate apparently served as H-acceptor since one mole of succinate was formed together with 4 moles of carbon dioxide for every three moles of tartrate consumed. Under aerobic conditions, the intact cell utilizes oxygen as the hydrogen acceptor. The oxygen uptake and carbon dioxide output observed when washed cells oxidize tartrate indicate that a considerable portion of the substrate is assimilated, but when DNP is added to the system the gas exchanges increase to the theoretical values for the complete oxidation of the tartrate (*272*). All of the microorganisms so far examined in detail appear to break down tartrate by the above route. However, Shilo & Stanier (*276*) who examined several *Pseudomonas* isolates, obtained preliminary indications that, in a few strains, breakdown occurs not through oxalacetate but through glyoxylate. It is possible that in these organisms tartrate is oxidized via oxaloglycollate and glyoxylate as in animal tissues (*277, 278*).

In considering the above work we have not so far considered the different optical isomers of tartaric acid. There is some confusion in the literature over the nomenclature of the two enantiomorphs of tartrate since, as pointed out by Shilo (*273*), both the dextro- and levorotatory forms of tartrate can be derived from D(+) glyceraldehyde, the usual reference standard. Consequently, following Shilo, we shall refer to the enantiomorphs as *d* and *l* according to their optical rotation.

The investigations mentioned above have been carried out principally with the *d*- or *meso*-isomers of tartrate but present evidence indicates that all three isomers are metabolized in the same way. However, the dehydrases catalysing the primary attack on tartrate are usually highly specific for one isomer. The most extensive investigation of these matters has been made by Shilo & Stanier (*276*), who isolated several fluorescent pseudomonads by enrichment in the presence of the various isomers of tartrate. Among these strains examples were found of organisms capable of utilizing all three, two, or only a single isomer. In agreement with previous observations on *Clostridium butyricum, Flavobacterium,* and *Pseudomonas putida* (*275, 279,* 280), the ability to attack tartrate was found to be inductive. The induction process was highly specific; washed cells only oxidized, without lag, the particular isomer utilized for growth and this held true even with one strain (*mmdl*) capable of growing on all three isomers. Shilo (*273*) assayed the tartaric dehydrase activity of cell extracts and found that their stereospecificity was absolute. Each preparation attacked only the isomer on which the cells were grown. In many cases, the other isomers were inhibitory and a stereochemical explanation was offered for the inhibition patterns observed.

The earlier observations of Martin & Foster (*279*) with a *Flavobacterium* and of la Rivière (280) with *P. putida,* are in agreement with the absolute stereospecificity of the dehydrases and of the enzymic induction but some exceptions to this rule have been observed. Shilo & Stanier (*276*) obtained one strain which, after growth on *d*-tartrate, showed an immediate oxidation of *l*-tartrate which, though weak, was regarded as significant. A more striking exception has been reported by Martin & Foster (281). They isolated a strain of *Pseudomonas fluorescens* which attacked all three isomers after growth on either *d*- or *l*- tartrate, although cells grown on *meso*-tartrate attacked only this isomer. The activities of extracts prepared from cells grown on *meso*- and *l*-tartrate reflected the activities of the whole cells.

An interesting finding came to light when Shilo & Stanier studied the activity of washed cells harvested from cultures after exhaustion of the tartrate (*276*). When the stationary phase was reached, tartrate was oxidized only after a lag period which increased with increasing age of culture. The tartaric dehydrase activity did not, however, parallel the oxidative activity of the whole cells. Thus, during the first four hours of the stationary phase, the ability of whole cells to oxidize tartrate immedi-

ately was lost completely whereas the dehydrase activity of extracts fell by only 50 per cent during the same period. The inactive whole cells still oxidized oxalacetate and pyruvate, indicating that the system lost was, in fact, concerned with the primary attack on tartrate. Shilo & Stanier suggested that an additional inducible system was required for transport of tartrate to the site of dehydration and this idea was confirmed by two lines of evidence. First, cells harvested from the stationary phase after growth on tartrate were irradiated briefly with ultraviolet light, a procedure known to prevent enzyme synthesis. Such cells did not regain the ability to oxidize tartrate when incubated with this substrate. Second, discrepancies were observed between the inhibitory effects of isomers on whole cells and on extracts. With one isolate, *d*-tartrate had no effect on the course of oxygen uptake when *meso*-tartrate was incubated with cells grown on the latter isomer and harvested at various times during the stationary phase. With extracts of the same organism, however, Shilo (*273*) observed that *d*-tartrate strongly inhibited the *meso*-dehydrase. Hence, it appears that the ability of cells to attack tartrate requires not only the induction of the specific dehydrase but also the induction of a transport system specific for the particular isomer. In the case of the *mmdl* strain, an *in vivo* inhibition by *d*-tartrate was observed. The authors suggest that in this case the transport system for the *d*-isomer may be constitutive.

*Transport.*—Cohen & Monod (*282*) have published an excellent review on the factors concerned with the entry of both substrates and analogues into the cell. It has been shown that highly specific proteins are involved in the process of transportation and, in the cases so far examined, these proteins are different from the enzymes which attack the substrates thus transported. The word permease has been coined for such proteins because ". . . it has the over-whelming advantage that its general meaning and scope are immediately understood." While the action of permeases has much in common with enzyme action, it still must be shown that a permease catalyzes a chemical change in its substrate and Cohen & Monod stress that one of the most immediate questions to be answered is whether or not the substrate is altered chemically during the actual process of entering the cell. The evidence presently available suggests that the substrate is in the same form inside as outside the cell.

Mitchell (*283*) has discussed the mechanism of transportation and has suggested that this process involves the formation of a covalent linkage between the substrate and the transporting system much in the same way as groups are believed to be attached to group-transfer enzymes. He postulates that when enzymes form part of the cell membrane they can effect the transport of their substrates; he also envisages specific proteins, with no enzymic activity in the accepted sense of the term, playing a part in transport. The term "translocase" is suggested for enzymes and specific proteins which mediate transport and "translocator" for the grouping to which the substrate is linked. There seems, at present, little choice between the names

permease and translocase. Since most of the experimental work which has led to the recognition of the fact that specific proteins are concerned with transport has been done by the French group, we think it advisable at present to use their term.

Specific permeases have been demonstrated in *E. coli* for β-galactosides, β-glucuronides, and amino acids. Pardee (284) has independently confirmed the work of the French group and from his work it would appear that a single, inducible permease is responsible for the transport of α- and β-galactosides. Rickenberg (285) has shown that protoplasts prepared from *E. coli* by the method devised by Repaske (286) concentrate β-galactosides as actively as whole cells and concludes that the permeases are not located in the cell wall or in that part which is removed in the process of making the protoplasts. The work of Shilo & Stanier (276) on the transport of tartrate has already been mentioned above.

## LITERATURE CITED


1. Campbell, J. J. R., *Ann. Rev. Microbiol.*, **8**, 71 (1954)
2. Delwiche, E. A., *Ann. Rev. Microbiol.*, **9**, 145 (1955)
3. O'Kane, D. J., *Ann. Rev. Microbiol.*, **10**, 275 (1956)
4. Wood, W. A., *Ann. Rev. Microbiol.*, **11**, 253 (1957)
5. Evans, W. C., *Ann. Repts. on Progr. Chem. (Chem. Soc., London), 1956*, **53**, 279 (1957)
6. Cohn, M., *Bacteriol. Rev.*, **21**, 140 (1957)
7. Vishniac, W., and Santer, M., *Bacteriol. Rev.*, **21**, 195 (1957)
8. Halvorson, H. O., and Church, B., *Bacteriol. Rev.*, **21**, 112 (1957)
9. Vishniac, W., Horecker, B. L., and Ochoa, S., *Advances in Enzymol.*, **19**, 1 (1957)
10. Barker, H. A., *Bacterial Fermentations* (John Wiley & Sons, Inc., New York, N.Y., 95 pp., 1957.
11. Quince, P., *A Midsummer Night's Dream* (Shakespeare, W., Act. V, Scene 1, Lines 108-11)
12. McGee, J., and Doudoroff, M., *J. Biol. Chem.*, **210**, 617 (1954)
13. Entner, N., and Doudoroff, M., *J. Biol. Chem.*, **196**, 853 (1952)
14. Gibbs, M., and DeMoss, R. D., *J. Biol. Chem.*, **207**, 689 (1954)
15. Sokatch, J. T., and Gunsalus, I. C., *J. Bacteriol.*, **73**, 452 (1957)
16. Sokatch, J. T., Prieto, A. P., and Gunsalus, I. C., *Bacteriol. Proc. (Soc. Am. Bacteriologists)*, 112 (1956)
17. Lampen, J. O., Gest, H., and Sowden, J. C., *J. Bacteriol.*, **61**, 97 (1951)
18. Rappoport, D. A., Barker, H. A., and Hassid, W. Z., *Arch. Biochem. Biophys.*, **31**, 326 (1951)
19. DeMoss, R. D., Bard, R. C., and Gunsalus, I. C., *J. Bacteriol.*, **62**, 499 (1951)
20. Gunsalus, I. C., and Gibbs, M., *J. Biol. Chem.*, **194**, 871 (1952)
21. Heath, E. C., Hurwitz, J., and Horecker, B. L., *J. Am. Chem. Soc.*, **78**, 5449 (1957)
22. Horecker, B. L., Heath, E. C., Hurwitz, J., and Ginsberg, A., *Federation Proc.*, **16**, 198 (1957)
23. Schramm, M., and Racker, E., *Nature*, **179**, 1349 (1957)
24. Dawes, E. A., and Foster, S. M., *Biochim. et Biophys. Acta.*, **22**, 253 (1956)
25. Burton, R. M., and Stadtman, E. R., *J. Biol. Chem.*, **202**, 873 (1953)
26. Buyze, G., van den Hamer, C. J. A., and de Haan, P. G., *Antonie van Leeuwenhoek, J. Microbiol. Serol.*, **23**, 345 (1957)
27. Nutting, L. A., and Carson, S. F., *J. Bacteriol.*, **63**, 575 (1952)
28. Neish, A. C., and Simpson, F. J., *Can. J. Biochem. and Physiol.*, **32**, 147 (1954)
29. Simpson, F. J., Kitch, B. B., and Wood, W. A., *Bacteriol. Proc. (Soc. Am. Bacteriologists)*, 111 (1956)
30. Simpson, F. J., and Wood, W. A., *J. Am. Chem. Soc.*, **78**, 5452 (1956)
31. Cynkin, M. A., and Gibbs, M., *Bacteriol. Proc. (Soc. Am. Bacteriologists)*, 122 (1957)
32. DeLey, J., and Doudoroff, M., *J. Biol. Chem.*, **227**, 745 (1957)
33. Palleroni, N. J., and Doudoroff, M., *J. Bacteriol.*, **74**, 180 (1957)
34. Kaplan, N. O., *Bacteriol. Rev.*, **19**, 234 (1955)
35. Mahler, H. R., *J. Biol. Chem.*, **206**, 13 (1954)
36. Pinchot, G. B., *J. Biol. Chem.*, **229**, 1 (1957)
37. Pinchot, G. B., *J. Biol. Chem.*, **229**, 25 (1957)

38. Brodie, A. F., and Gray, C. T., *J. Biol. Chem.*, **219**, 853 (1956)
39. Brodie, A. F., Weber, M. M., and Gray, C. T., *Biochim. et Biophys. Acta*, **25**, 447 (1957)
40. Weber, M. M., and Brodie, A. F., *Biochim. et Biophys. Acta*, **25**, 447 (1957)
41. Brodie, A. F., and Gray, C. T., *Bacteriol. Proc. (Soc. Am. Bacteriologists)*, 110 (1957)
42. Wosilait, W. D., and Nason, A., *J. Biol. Chem.*, **208**, 785 (1954)
43. Hartman, P. E., Brodie, A. F., and Gray, C. T., *J. Bacteriol.*, **74**, 319 (1957)
44. Bruemmer, J. H., Wilson, P. W., Glenn, J. L., and Crane, F. L., *J. Bacteriol.*, **73**, 113 (1957)
45. Tissières, A., Hovenkamp, H. G., and Slater, E. C., *Biochim. et Biophys. Acta*, **25**, 336 (1957)
46. Tissières, A., and Slater, E. C., *Nature*, **176**, 736 (1955)
47. Rose, I. A. and Ochoa, S., *J. Biol. Chem.*, **220**, 307 (1956)
48. Josten, J. L., and Repaske, R., *Bacteriol. Proc. (Soc. Am. Bacteriologists)*, 109 (1957)
49. Klungsöyr, L., King, T. E., and Cheldelin, V. H., *J. Biol. Chem.*, **227**, 135 (1957)
50. King, T. E., and Cheldelin, V. H., *J. Biol. Chem.*, **224**, 579 (1957)
51. Smith, L., *Bacteriol. Rev.*, **18**, 106 (1954)
52. Monod, J., *Recherches sur la croissance des cultures bactériennes.* (Librairie scientifique, Hermann & Cie, Paris, 210 pp., 1942)
53. Bauchop, T., *J. Gen. Microbiol.*, **18**, vii (1958)
54. Knivett, V. A., *Biochem. J.*, **56**, 602 (1954)
55. Krebs, H. A., and Kornberg, H. L., *Energy Transformations in Living Matter.* (Springer-Verlag OHG, Berlin-Charlottenburg, 87 pp., 1957), (reprinted from *Ergeb. Physiol., biol. Chem. u exptl. Pharmakol.*, **49**, 212, 1957)
56. Walker, T. K., and Wright, H. B., *Arch. Biochem. Biophys.*, **69**, 361 (1957)
57. Steel, R., and Walker, T. K., *Nature*, **180**, 201 (1957)
58. Schramm, M., and Hestrin, S., *J. Gen. Microbiol.*, **11**, 123 (1954)
59. Steel, R., and Walker, T. K., *J. Gen. Microbiol.*, **17**, 12 (1957)
60. Steel, R., and Walker, T. K., *J. Gen. Microbiol.*, **17**, 445 (1957)
61. Shimwell, J. H., *J. Inst. Brewing*, **65**, 44 (1957)
62. Shimwell, J. H., *J. Inst. Brewing*, **65**, 54 (1957)
63. Shimwell, J. H., *Antonie van Leewenhoek. J. Microbiol. Serol.*, **23**, 235 (1957)
64. Minor, F. W., Greathouse, G. A., Shirk, H. G., Schwartz, A. M., and Harris, M., *J. Am. Chem. Soc.*, **76**, 1658 (1954)
65. Minor, F. W., Greathouse, G. A., and Shirk, H. G., *J. Am. Chem. Soc.*, **77**, 1244 (1955)
66. Greathouse, G. A., *J. Am. Chem. Soc.*, **79**, 4505 (1957)
67. Greathouse, G. A., *J. Am. Chem. Soc.*, **79**, 4503 (1957)
68. Schramm, M., and Hestrin, S., *Biochem. J.*, **56**, 163 (1954)
69. Hestrin, S., and Schramm, M., *Biochem. J.*, **58**, 345 (1954)
70. Schramm, M., Gromet, Z., and Hestrin, S., *Biochem. J.*, **67**, 669 (1957)
71. Gromet, Z., Schramm, M., and Hestrin, S., *Biochem. J.*, **67**, 679 (1957)
72. Schramm, M., Gromet, Z., and Hestrin, S., *Nature*, **179**, 28 (1957)
73. Colvin, J. R., *Arch. Biochem. Biophys.*, **70**, 294 (1957)
74. Glaser, L., *Biochim. et Biophys. Acta*, **25**, 436 (1957)

75. Colvin, J. R., Bayley, S. T., and Beer, M., *Biochim. et Biophys. Acta*, **23**, 652 (1957)
76. Sijpesteijn, A. K., *J. Gen. Microbiol.*, **5**, 869 (1951)
77. Hungate, R. E., *Bacteriol. Rev.*, **14**, 1 (1950)
78. Baker, F., and Harris, S. T., *Nutrition Abstr. & Revs.*, **17**, 1 (1947)
79. Winogradsky, S., *Microbiologie du sol* (Masson et Cie, Paris, France, 861 pp., 1949)
80. Stanier, R. Y., *Bacteriol. Rev.*, **6**, 143 (1942)
81. Halliwell, G., *J. Gen. Microbiol.*, **17**, 166 (1957)
82. Sih, C. J., and McBee, R. H., *Bacteriol. Proc.* (*Soc. Am. Bacteriologists*), 126 (1955)
83. Sih, C. J., Nelson, N. M., and McBee, R. H., *Science*, **126**, 116 (1957)
84. Nelson, N. M., and McBee, R. H., *Bacteriol. Proc.* (*Soc. Am. Bacteriologists*), 121 (1957)
85. Crook, E. M., and Stone, B. A., *Biochem. J.*, **65**, 1 (1957)
86. Halliwell, G., *J. Gen. Microbiol.*, **17**, 166 (1957)
87. Grimes, R. W., Duncan, C. W., and Hoppert, C. A., *Arch. Biochem. Biophys.*, **68**, 412 (1957)
88. Sison, B. C., Schubert, W. J., and Nord, F. F., *Arch. Biochem. Biophys.*, **68**, 502 (1957)
89. Mandels, M., and Reese, E. T., *J. Bacteriol.*, **73**, 269 (1957)
90. Cabib, E., Leloir, L. F., and Cardini, C. E., *J. Biol. Chem.*, **203**, 1055 (1953)
91. Storey, I. D. E., and Dutton, G. J., *Biochem. J.*, **59**, 279 (1955)
92. Smith, E. E. B., and Mills, G. T., *Biochim. et Biophys. Acta*, **13**, 386 (1954)
93. Smith, E. E. B., Mills, G. T., and Harper, E. M., *J. Gen. Microbiol.*, **16**, 426 (1957)
94. Smith, E. E. B., Mills, G. T., Harper, E. M., and Galloway, B., *J. Gen. Microbiol.*, **17**, 437 (1957)
95. Smith, E. E. B., Mills, G. T., and Harper, E. M., *Biochim. et Biophys. Acta*, **23**, 622 (1957)
96. Whistler, R. L., and Olson, E. J., *Advances in Carbohydrate Chem.*, **12**, 299 (1957)
97. Cifonelli, J. A., and Dorfman, A., *J. Biol. Chem.*, **228**, 547 (1957)
98. Strange, R. E., and Powell, J. F., *Biochem. J.*, **58**, 80 (1954)
99. Strange, R. E., and Dark, F. A., *Nature*, **177**, 187 (1956)
100. Strange, R. E., *Biochem. J.*, **64**, 23p (1956)
101. Kent, L. H., *Biochem. J.*, **67**, 5p (1957)
102. Park, J. T., and Strominger, J. L., *Science*, **125**, 99 (1957)
103. Park, J. T., *J. Biol. Chem.*, **194**, 897 (1952)
104. Cumming, C. S., and Harris, H., *J. Gen. Microbiol.*, **14**, 583 (1956)
105. Work, E., *Nature*, **179**, 841 (1957)
106. Salton, M. R. J., *Bacteriol. Rev.*, **21**, 82 (1957)
107. Glaser, L., and Brown, D. H., *Biochim. et Biophys. Acta*, **23**, 449 (1957)
108. Glaser, L., and Brown, D. H., *J. Biol. Chem.*, **28**, 729 (1957)
109. Doetsch, R. N., Howard, B. H., Mann, S. O., and Oxford, A. E., *J. Gen. Microbiol.*, **16**, 156 (1957)
110. Van Sumere, C. F., and Shu, P., *Can. J. Biochem. and Physiol.*, **35**, 445 (1957)
111. Loewenberg, J. R., and Reese, E. T., *Can. J. Microbiol.*, **3**, 643 (1957)

112. Henis, Y., *J. Gen. Microbiol.*, **15**, 462 (1956)
113. Avigad, G., and Feingold, D. S., *Arch. Biochem. Biophys.*, **70**, 178 (1957)
114. Feingold, D. S., Avigad, G., and Hestrin, S., *Biochem. J.*, **64**, 351 (1956)
115. Dedonder, R., and Noblesse, M., *Ann. inst. Pasteur*, **85**, 356 (1953)
116. French, D., and Wild, G. M., *J. Am. Chem. Soc.*, **75**, 2612 (1953)
117. Lindeberg, G., *Nature*, **180**, 1141 (1957)
118. Avigad, G., *J. Biol. Chem.*, **229**, 121 (1957)
119. Holme, T., Laurent, T., and Palmstierna, H., *Acta Chem. Scand.*, **11**, 757 (1957)
120. Holme, T., *Acta Chem. Scand.*, **11**, 763 (1957)
121. Eliasson, N. A., Hammarsten, E., Palmistierna, H., and Äquist, S., *Acta Chem. Scand.*, **11**, 1381 (1957)
122. Palmstierna, H., and Stjernholm, R., *Acta Chem. Scand.*, **11**, 900 (1957)
123. Gary, N. D., Kupferberg, L. L., Anderson, R. E., Harein, P. K., and Klausmeier, R. E., *J. Bacteriol.*, **73**, 632 (1957)
124. Rogers, H. J., *J. Gen. Microbiol.*, **16**, 22 (1957)
125. Monod, J., *Ann. inst. Pasteur*, **79**, 390 (1950)
126. Howard, R. B., *Biochem. J.*, **67**, 643 (1957)
127. Pazur, J. H., Budovich, T.. Shuey, E. W., and Georgi, C. E., *Arch. Biochem. Biophys.*, **70**, 419 (1957)
128. Jarvis, F. G., and Johnson, M. J., *J. Am. Chem. Soc.*, **71**, 4124 (1949)
129. Hauser, G., and Karnovsky, M. L., *J. Biol. Chem.*, **224**, 91 (1957)
130. Rush, D., Karibian, D., Karnovsky, M. L., and Magasanik, B., *J. Biol. Chem.*, **226**, 891 (1957)
131. Hauser, G., and Karnovsky, M. L., *Federation Proc.*, **16**, 193 (1957)
132. Wilkinson, J. F., *Nature*, **180**, 995 (1957)
133. Heath, E. C., and Roseman, S., *Bacteriol. Proc.* (*Soc. Am. Bacteriologists*), 123 (1957)
134. Segal, S., and Topper, Y. J., *Biochim. et Biophys. Acta*, **25**, 419 (1957)
135. MacLennan, A. P., and Davies, D. A. L., *Biochem. J.*, **66**, 562 (1957)
136. Davies, D. A. L., *Nature*, **180**, 1129 (1957)
137. MacLennan, A. P., and Randle, C. J. M., *Nature*, **180**, 1045 (1957)
138. Walker, N., and Evans, W. C., *Biochem. J.*, **52**, xxiii (1952)
139. Roof, B. S., Lannon, T. J., and Turner, J. C., *Proc. Soc. Exptl. Biol. Med.*, **84**, 38 (1953)
140. Gross, S. R., Gafford, R. D., and Tatum, E. L., *J. Biol. Chem.*, **219**, 781 (1956)
141. Stanier, R. Y., and Ingraham, J. L., *J. Biol. Chem.*, **210**, 799 (1954)
142. Ottey, L., and Tatum, E. L., *J. Biol. Chem.*, **223**, 307 (1956)
143. Crandall, D. I., *A Symposium on Amino-Acid Metabolism*, 867 ( McElroy, W. D., and Glass, H. B., Eds., Johns Hopkins Press, Baltimore, Md., 1048 pp., 1955)
144. Dagley, S., and Patel, M. D., *Biochem. J.*, **66**, 227 (1957)
145. Hayaishi, O., Katagiri, M., and Rothberg, S., *J. Am. Chem. Soc.*, **77**, 5450 (1955)
146. Behrman, E. J., and Stanier, R. Y., *J. Biol. Chem.*, **228**, 923 (1957)
147. Pinsky, A., and Michaelis, M., *Biochem. J.*, **52**, 33 (1952)
148. Harary, I., *J. Biol. Chem.*, **227**, 815 (1957)
149. Yasunobu, K. T., and Norris, E. R., *J. Biol. Chem.*, **227**, 473 (1957)

150. MacDonald, D. L., Stanier, R. Y., and Ingraham, J. L., *J. Biol. Chem.*, **210**, 809 (1954)
151. Sistrom, W. R., and Stanier, R. Y., *J. Biol. Chem.*, **210**, 821 (1954)
152. Evans, W. C., Smith, B. S. W., Linstead, R. P., and Elvidge, J. A., *Nature*, **168**, 772 (1951)
153. Tatum, E. L., and Gross, S. R., *J. Biol. Chem.*, **219**, 797 (1956)
154. Elvidge, J. A., Linstead, R. P., Orkin, B. A., Sims, P., Baer, H., and Pattison, D. B., *J. Chem. Soc.*, 2228 (1950)
155. Kilby, B. A., *Biochem. J.*, **49**, 671 (1951)
156. Happold, F. C., and Key, A., *J. Hyg.*, **32**, 573 (1932)
157. Katagiri, M., and Hayaishi, O., *J. Biol. Chem.*, **226**, 439 (1957)
158. Smith, B. S. W., Jones, J. D., and Evans, W. C., *Biochem. J.*, **50**, xxviii (1952)
159. Tausson, W. O., *Planta*, **4**, 214 (1927)
160. Tausson, W. O., *Planta*, **5**, 239 (1928)
161. Gray, P. H., and Thornton, H. G., *Zentr. Bakteriol. Parasitenk. Abt II*, **73**, 74 (1928)
162. Walker, N., and Wiltshire, G. H., *J. Gen. Microbiol.*, **8**, 273 (1953)
163. Trecanni, V., Walker, N., and Wiltshire, G. H., *J. Gen. Microbiol.*, **11**, 341 (1954)
164. Strawinski, R. J., and Stone, R. W., *Can. J. Microbiol.*, **1**, 206 (1954)
165. Murphy, J. F., and Stone, R. W., *Can. J. Microbiol.*, **1**, 579 (1955)
166. Klausmeier, R. E., and Strawinski, R. J., *J. Bacteriol.*, **73**, 461 (1957)
167. Rogoff, M. H., and Wender, I., *J. Bacteriol.*, **73**, 264 (1957)
168. Tausson, W. O., *Neftyanoe Khorz*, **14**, 220 (1928)
169. Rogoff, M. H., and Wender, I., *J. Bacteriol.*, **74**, 108 (1957)
170. Henderson, M. E. K., and Farmer, V. C., *J. Gen. Microbiol.*, **12**, 37 (1955)
171. Henderson, M. E. K., *J. Gen. Microbiol.*, **14**, 684 (1956)
172. Sowden, F. J., and Atkinson, H. J., *Soil Sci.*, **68**, 433 (1949)
173. Henderson, M. E. K., *J. Gen. Microbiol.*, **16**, 686 (1957)
174. Nutman, P. S., Thornton, H. G., and Quastel, J. H., *Nature*, **155**, 498 (1945)
175. Jensen, H. L., and Peterson, H. I., *Acta Agr. Scand.*, **2**, 215 (1952)
176. Evans, W. C., and Smith, B. S. W., *Biochem. J.*, **57**, xxx (1954)
177. Audus, L. J., and Symonds, K. V., *Ann. Appl. Biol.*, **42**, 174 (1955)
178. Rogoff, M. H., and Reid, J. J., *J. Bacteriol.*, **71**, 303 (1956)
179. Steenson, T. I., and Walker, N., *Plant and Soil*, **8**, 17 (1956)
180. Steenson, T. I., and Walker, N., *J. Gen. Microbiol.*, **16**, 146 (1957)
181. Walker, N., and Wiltshire, G. H., *J. Gen. Microbiol.*, **12**, 478 (1955)
182. Krebs, H. A., Gurin, S., and Eggleston, L. V., *Biochem. J.*, **51**, 614 (1952)
183. Roberts, R. B., Abelson, P. H., Cowie, D. B., Bolton, E. T., and Britten, R. J., *Studies of Biosynthesis in* Escherichia coli (Carnegie Institution of Washington, 521 pp., 1955)
184. Cutinelli, C., Ehrensvärd, G., Reio, L., Saluste, E., and Stjernholm, R., *Acta Chem. Scand.*, **5**, 353 (1951)
185. Ehrensvärd, G., Reio, L., Saluste, E., and Stjernholm, R., *J. Biol. Chem.*, **189**, 93 (1951)
186. Cutinelli, C., Ehrensvärd, G., Högström, G., Reio, L., Saluste, E., and Stjernholm, R., *Arkiv. Kemi*, **3**, 501 (1951)
187. Abelson, P. H., and Vogel, H. J., *J. Biol. Chem.*, **213**, 355 (1955)
188. Moses, V., *J. Gen. Microbiol.*, **16**, 534 (1957)

189. Ochoa, S., Mehler, A. H., and Kornberg, A., *J. Biol. Chem.*, **174**, 979 (1948)
190. Korkes, S., del Campillo, A., and Ochoa, S., *J. Biol. Chem.*, **187**, 891 (1950)
191. Utter, M. F., and Kurahashi, K., *J. Biol. Chem.*, **207**, 821 (1954)
192. Wood, H. G., and Werkman, C. H., *Biochem. J.*, **32**, 1262 (1938)
193. Wood, H. G., and Werkman, C. H., *Biochem. J.*, **34**, 129 (1940)
194. Johns, A. T., *J. Gen. Microbiol.*, **5**, 326 (1951)
195. Johns, A. T., *J. Gen. Microbiol.*, **5**, 337 (1951)
196. Tomlinson, N., *J. Biol. Chem.*, **209**, 597 (1954)
197. Abelson, P. H., Bolton, E. T., Britten, R. J., Cowie, D. B., and Roberts, R. B., *Yearbook Carnegie Inst. Wash.*, **53**, 77 (1954)
198. Utter, M. F., Lipmann, F., and Werkman, C. H., *J. Biol. Chem.*, **158**, 521 (1948)
199. Strecker, H. J., Wood, H. G., and Krampitz, L. O., *J. Biol. Chem.*, **182**, 525 (1950)
200. Wolfe, R. S., and O'Kane, D. J., *J. Biol. Chem.*, **215**, 637 (1953)
201. Novelli, G. D., *Biochim. et Biophys. Acta*, **18**, 594 (1955)
202. Thunberg, T., *Skand. Arch. Physiol.*, **40**, 1 (1920)
203. Foster, J. W., Carson, S. F., Anthony, D. S., Davis, J. B., Jefferson, W. E., and Long, M. V., *Proc. Natl. Acad. Sci. U.S.*, **35**, 663 (1949)
204. Foster, J. W., and Carson, S. F., *Proc. Natl. Acad. Sci. U.S.*, **36**, 219 (1950)
205. Barron, E. S. G., and Ghiretti, F., *Biochim. et Biophys. Acta*, **12**, 239 (1953)
206. Seaman, G. R., and Nashke, M. D., *J. Biol. Chem.*, **217**, 1 (1955)
207. Seaman, G. R., *J. Biol. Chem.*, **228**, 149 (1957)
208. Wong, D. T. O., and Ajl, S. J., *J. Am. Chem. Soc.*, **78**, 3230 (1956)
209. Wong, D. T. O., and Ajl, S. J., *Science*, **126**, 1013 (1957)
210. Ochoa, S., Stern, J. R., and Schneider, M. C., *J. Biol. Chem.*, **193**, 691 (1951)
211. Krakow, G., and Barkulis, S. S., *Biochim. et Biophys. Acta*, **21**, 593 (1956)
212. Tolbert, N. E., and Cohen, M. S., *J. Biol. Chem.*, **204**, 639 (1953)
213. Franke, W., and Schulz, I., *Hoppe-Seyler's Z. physiol. Chem.*, **303**, 30 (1956)
214. Kornberg, H. L., and Madsen, N. B., *Biochim. et Biophys. Acta*, **24**, 651 (1957)
215. Kornberg, H. L., and Madsen, N. B., *Biochem. J.*, **66**, 13p (1957)
216. Kogut, M., and Podoski, E. P., *Biochem. J.*, **55**, 800 (1953)
217. Jones, M. E., and Lipmann, F., *Methods in Enzymology*, **1**, 585 (Kaplan, N. O., and Colowick, S. P., Eds., Academic Press, Inc., New York, N.Y., 835 pp., 1955)
218. Campbell, J. J. R., Smith, R. A., and Eagles, B. A., *Biochim. et Biophys. Acta*, **11**, 594 (1953)
219. Olson, J. A., *Nature*, **174**, 695 (1954)
220. Smith, R. A., and Gunsalus, I. C., *J. Am. Chem. Soc.*, **76**, 5002 (1954)
221. Saz, H. J., and Hillary, E. P., *Biochem. J.*, **62**, 563 (1956)
222. Kornberg, H. L., and Krebs, H. A., *Nature*, **179**, 988 (1957)
223. Kornberg, H. L., *Biochim. et Biophys. Acta*, **25**, 200 (1957)
224. Kornberg, H. L., *Biochem. J.*, **66**, 13p (1957)
225. Dagley, S., and Patel, M. D., *Biochim. et Biophys. Acta*, **16**, 418 (1955)
226. Callely, A. G., Dagley, S., and Hodgson, B., *Biochem. J.*, **66**, 47 (1957)
227. Smith, R. A., and Gunsalus, I. C., *Nature*, **175**, 774 (1955)
228. Smith, R. A., and Gunsalus, I. C., *J. Biol. Chem.*, **229**, 305 (1957)
229. Wong, D. T. O., and Ajl, S. J., *Nature*, **176**, 970 (1955)
230. Bachrach, U., *J. Gen. Microbiol.*, **17**, 1 (1957)

231. Jayasuriya, G. C. N., *Biochem. J.*, **58,** xxxi (1954)
232. Jayasuriya, G. C. N., *Biochem. J.*, **64,** 469 (1956)
233. Anderson, E. H., *J. Gen. Physiol.*, **28,** 297 (1945)
234. Weimberg, R., and Doudoroff, M., *J. Biol. Chem.*, **217,** 607 (1955)
235. Swim, H. E., and Krampitz, L. O., *J. Bacteriol.*, **67,** 419 (1954)
236. Swim, H. E., and Krampitz, L. O., *J. Bacteriol.*, **67,** 426 (1954)
237. Saz, H. J., and Krampitz, L. O., *J. Bacteriol.*, **67,** 409 (1954)
238. DeMoss, J. A., and Swim, H. E., *J. Bacteriol.*, **74,** 445 (1957)
239. Eaton, N. R., and Klein, H. P., *Biochem. J.*, **67,** 373 (1957)
240. Horecker, B. L., and Mehler, A. H., *Ann. Rev. Biochem.*, **24,** 207 (1955)
241. Fisher, K. W., *J. Gen. Microbiol.*, **16,** 120 (1957)
242. Hayes, W., *J. Gen. Microbiol.*, **16,** 97 (1957)
243. Fisher, K. W., *J. Gen. Microbiol.*, **16,** 136 (1957)
244. Bentley, R. L., and Thiessen, C. P., *J. Biol. Chem.*, **226,** 673 (1957)
245. Bentley, R. L., and Thiessen, C. P., *J. Biol. Chem.*, **226,** 689 (1957)
246. Bentley, R. L., and Thiessen, C. P., *J. Biol. Chem.*, **226,** 703 (1957)
247. Ogston, A. G., *Nature*, **162,** 963 (1948)
248. Magasanik, B., Brooke, M. S., and Karibian, D., *J. Bacteriol.*, **66,** 611 (1953)
249. Karnovsky, M. L., Hauser, G., and Elwyn, O., *J. Biol. Chem.*, **226,** 881 (1957)
250. Cummins, J. T., King, T. E., and Cheldelin, V. H., *J. Biol. Chem.*, **224,** 323 (1957)
251. Cummins, J. T., Cheldelin, V. H., and King, T. E., *J. Biol. Chem.*, **226,** 301 (1957)
252. Umbarger, H. E., Brown, B., and Eyring, E. J., *J. Am. Chem. Soc.*, **79,** 2980 (1957)
253. Willson, C. P., and Adelberg, E. A., *J. Biol. Chem.*, **229,** 1011 (1957)
254. Juni, E., *J. Biol. Chem.*, **195,** 715 (1952)
255. Juni, E., and Heym, G. A., *J. Bacteriol.*, **74,** 757 (1957)
256. McIntosh, E. N., Purko, M., and Wood, W. A., *J. Biol. Chem.*, **228,** 449 (1957)
257. Woods, D. D., and Clifton, C. E., *Biochem. J.*, **32,** 345 (1938)
258. Morrison, J. F., *Biochem. J.*, **58,** 685 (1954)
259. Peck, H. D., Jr., and Gest, H., *J. Bacteriol.*, **73,** 706 (1957)
260. Gest, H., and Peck, H. D., Jr., *J. Bacteriol.*, **70,** 326 (1955)
261. Woods, D. D., *Biochem. J.*, **30,** 515 (1936)
262. Peck, H. D., Jr., and Gest, H., *J. Bacteriol.*, **73,** 569 (1957)
263. Hauge, J. G., *Biochim. et Biophys. Acta*, **25,** 148 (1957)
264. Hug, D. H., and Sagers, R. D., *Bacteriol. Proc.* (*Soc. Am. Bacteriologists*), 111 (1957)
265. Sutton, W. B., *J. Biol. Chem.*, **226,** 395 (1957)
266. Sutton, W. B., and Hayaishi, O., *J. Am. Chem. Soc.*, **79,** 4809 (1957)
267. Hayaishi, O., Shimazono, H., Katagiri, M., and Saito, Y., *J. Am. Chem. Soc.*, **78,** 5126 (1956)
268. Shimazono, H., and Hayaishi, O., *J. Biol. Chem.*, **227,** 151 (1957)
269. Jakoby, W. B., Ohmura, E., and Hayaishi, O., *J. Biol. Chem.*, **222,** 435 (1956)
270. Barker, H. A., *Proc. Acad. Sci. Amsterdam*, **39,** 674 (1936)
271. Nomura, M., *Chem. Abstr.*, **48,** 14878 (1955)
272. la Rivière, J. W. M., *Biochim. et Biophys. Acta*, **21,** 190 (1956)
273. Shilo, M., *J. Gen. Microbiol.*, **16,** 472 (1957)
274. Krampitz, L. O., and Lynen, F., *Federation Proc.*, **15,** 292 (1956)

275. Tabachnick, J., and Vaughn, R. H., *J. Bacteriol.*, **56**, 435 (1948)
276. Shilo, M., and Stanier, R. Y., *J. Gen. Microbiol.*, **16**, 482 (1957)
277. Kun, E., *J. Biol. Chem.*, **221**, 223 (1956)
278. Kun, E., and Hernandez, M. G., *J. Biol. Chem.*, **218**, 201 (1956)
279. Martin, W. R., and Foster, J. W., *J. Bacteriol.*, **70**, 405 (1955)
280. la Rivière, J. W. M., *Biochim. et Biophys. Acta*, **22**, 206 (1956)
281. Martin, W. R., and Foster, J. W., *J. Bacteriol.*, **73**, 683 (1957)
282. Cohen, G. N., and Monod, J., *Bacteriol. Rev.*, **21**, 169 (1957)
283. Mitchell, P., *Nature*, **180**, 134 (1957)
284. Pardee, A. B., *J. Bacteriol.*, **73**, 376 (1957)
285. Rickenberg, H. V., *Biochim. et Biophys. Acta*, **25**, 206 (1957)
286. Repaske, R., *Biochim. et Biophys. Acta*, **22**, 189 (1956)

# INORGANIC NITROGEN METABOLISM[1,2]

By Alvin Nason
*McCollum-Pratt Institute, The Johns Hopkins University, Baltimore, Maryland*
*and*
Hajime Takahashi
*Institute of Applied Microbiology, University of Tokyo, Bunkyo-Ku, Tokyo, Japan*

The ultimate source of nitrogen for all forms of life is inorganic nitrogen. The nitrogen atom has a variety of oxidation states ranging from the disputed oxidation level of plus six as represented by the presumed short half-lived $NO_3$ [Wells (1)] through the oxidation states of plus five ($N_2O_5$ or its hydrated form, $HNO_3$); plus four ($NO_2$); plus three ($N_2O_3$ or its hydrated form, $HNO_2$), plus two (NO); plus one ($N_2O$, [HNO], $H_2N_2O_2$; and $NO_2{\cdot}NH_2$ -nitrous oxide, nitroxyl, hyponitrous acid [the dimer of the hypothetical nitroxyl], and nitramide, respectively); zero ($N_2$); minus one ($NH_2OH$); minus two ($NH_2NH_2$); and minus three ($NH_3$).

With the exception of the controversial plus six oxidation state, all of the above forms have been implicated in inorganic nitrogen metabolism with either intact organisms or cell-free preparations. The important biological questions concerning most of the inorganic nitrogen forms are: (*a*) what are the mechanisms of their absorption and stepwise conversion to either the organic form as represented by amino acids and proteins, or to the more oxidized states as occurs in nitrification; and (*b*) what other functions do they perform in the metabolism of the living cell, for example, in terminal electron transport and possibly in coupled phosphorylation. In recent years, the various aspects of inorganic nitrogen metabolism have received increasing attention with notable results. Although past volumes of this *Annual Review of Microbiology* contained articles concerned with only one phase of the subject, namely, nitrogen fixation [Virtanen (2); Wilson & Burris (3); Lockhead (4)], there were no papers dealing with the broad area of inorganic nitrogen function. The present reviewers have therefore given their attention to the whole field of inorganic nitrogen metabolism. This includes nitrogen nutrition, nitrate respiration, nitrate assimilation, denitrification, nitroaryl reduction, nitrification, and nitrogen

[1] The survey of the literature pertaining to this review was concluded in January, 1958.

Contribution No. 230 of the McCollum-Pratt Institute.

[2] The following abbreviations are used: ATP (adenosine triphosphate); FAD, FADH (flavin adenine dinucleotide and reduced form); FMN, FMNH (flavin mononucleotide and reduced form); DPN, DPNH (diphosphopyridine nucleotide and reduced form); TPN, TPNH (triphosphopyridine nucleotide and reduced form).

fixation. No attempt has been made to cover all studies dealing with inorganic nitrogen. Instead, the emphasis has been placed on those papers having a direct bearing on the overall pathways and on the mechanisms of action of the steps concerned. The literature coverage includes contributions made to the field since 1955. The important aspects up to that time were comprehensively treated in the McCollum-Pratt *Symposium on Inorganic Nitrogen Metabolism* (5). In a number of cases earlier material has been included where it seemed necessary for a logical presentation of the subject. While the present treatment is concerned primarily with microorganisms, there has been no hesitation in discussing those higher plants and animals which contain systems bearing on the topics under consideration.

## Absorption and Transport of Inorganic Nitrogen

There is growing evidence that the passage into and the accumulation within the microbial cell of inorganic nitrogen compounds against a concentration gradient requires an active transport mechanism. DeTurk (6) reported that the assimilation of ammonia by washed cells of *Pseudomonas aeruginosa* was inhibited by streptomycin and obligatorily linked with the oxidation of added substrates of the tricarboxylic acid cycle. He claimed that studies with broken cell suspensions indicated two enzymatic reactions which probably function in assimilation: (*a*) glutamate formation from ammonium sulfate and ketoglutarate, and (*b*) aspartate formation from fumarate or malate. These reactions were not inhibited by streptomycin and occurred under anaerobic conditions in cell-free extracts, whereas they did not take place in whole cells under similar conditions. The organic acids permeated the cells under anaerobic conditions. It was therefore suggested that ammonia gained access to the cell by a streptomycin-sensitive, active transport mechanism which required simultaneous oxidation. Farkas-Hinsley & Artman (7) showed that while the kinetics of nitrate reduction by nonproliferating suspensions of old *Escherichia coli* cells with and without nitrate was suggestive of an adaptive system, it could be ascribed instead to a permeability barrier in old cells towards nitrate. This was supported by the observed high rate of nitrate reduction by cell extracts and the fact that nonproliferating young cells reduced nitrate at the same rapid rate regardless of the previous presence or absence of nitrate in the growth medium. Macmillan (8) demonstrated with nitrate-starved mycelia of *Scopulariopsis brevicaulis* that subsequent $NO_3^-$ accumulation without added glucose occurred in two phases. The first was a passive process which was not inhibited by cyanide and lasted 3 to 6 hr. No accumulation of $NO_3^-$ took place during this time, the rate of entry depending upon the external concentration. Most of the nitrate was presumed to be held by adsorption in the fungal hyphae since little was found in fluid expressed from the mycelia. During the second phase, nitrate accumulation occurred and apparently required respiratory energy in view of its inhibition by cyanide and azide. The rate of entry was slower and independent of external nitrate

concentration. If glucose was supplied together with $NO_3^-$, nitrate uptake was stimulated but no accumulation resulted since nitrate was being assimilated as rapidly as it entered. The process of nitrate entry into fungi is essentially similar to that in higher plants. The results are consistent with the evidence for other types of microbial cells such as *Staphylococcus* [Britten (9)] and *E. coli* [Cowie & Roberts (10)] which indicated that the initial entry of ions involved passive equilibration of part of the cell volume with the external concentration.

Morton & Macmillan (11) pointed out that most fungi utilize ammonium salts as a suitable nitrogen source and many but not all use nitrate as well. Fungi are also capable of growing on various amino acids, proteins, and other organic nitrogen compounds, while a relatively small number, usually parasitic forms, require organic nitrogen and are unable to grow on inorganic nitrogen. They demonstrated that in a number of fungi the assimilation of ammonium ion prevented the simultaneous assimilation of nitrate but not of nitrite. Ammonia inhibited the reduction of nitrate to nitrite. These fungi were shown not to utilize nitrate in early stages of growth, nitrate uptake beginning only after the ammonia in the medium had fallen to a low level. In a second group of fungi the inhibitory effect of $NH_3$ was less extreme, with a 42 to 82 per cent decrease in nitrate uptake during the first 5 hr. In a third group, including *Aspergillus niger* and *Penicillium griseofulvum*, nitrate is used simultaneously with ammonia "although relatively more slowly." Finally, there are certain fungi which even assimilate nitrate exclusively [Morton (12)]. In examining the inhibitory effect of ammonia on nitrate assimilation as indicated for the first group of fungi above, Morton (12) observed that the effect was not caused by a competition for hydrogen donors or coenzymes. Using *Scopulariopsis brevicaulis*, *Penicillium griseofulvum*, and *Myrothecium verrucaria*, he verified the presence of a TPN-nitrate reductase as found by Nason & Evans (13) in *Neurospora* and indicated the inhibition by ammonia was exerted on the formation and stability of this enzyme. This was also shown with an *Aspergillus nidulans* mutant which used ammonia but not nitrate or nitrite since it was genetically blocked in the reduction of nitrite. The mutant was grown on a casein hydrolysate nitrogen source with nitrate added for nitrate reductase formation. One hour after exposure of the mycelium to a mixture of nitrate and ammonia, nitrate reductase activity fell to zero. The effect of ammonia in lowering the activity of the enzyme was linked to ammonia assimilation since (*a*) $NH_4^+$ did not inhibit the cell-free system; (*b*) the decrease in activity *in vivo* involved a time element; and (*c*) oxygen was necessary for inactivation by ammonia and for assimilation of ammonia. If ammonia assimilation was prevented by the absence of oxygen or the addition of iodoacetate, there was no fall in nitrate reductase in the presence of ammonium ion. Incubation of mycelia without glucose caused a practically complete loss of nitrate reductase in 2 to 3 hr. in the presence of oxygen, with no recovery of the enzyme upon addition of glucose unless

oxygen was also present. Although it is not yet clear how the assimilation of ammonia affects nitrate reductase, the use of the latter's apoenzyme for the synthesis of the ammonia assimilation system is a distinct possibility. An inhibitory effect of ammonium sulfate on the formation of nitrate reductase in *E. coli* has also been indicated by Wainwright & Nevill (14). Working with *Scopulariopsis brevicaulis,* Macmillan (15) showed that ammonia entered the hypha as undissociated molecules by passive diffusion dependent on the concentration gradient; and that the level of ammonia in the cell was independent of respiratory energy, in contrast to the energy requirement for nitrate accumulation. The need for oxygen in ammonia assimilation with the same fungus [Morton (12)] pointed to a respiratory requirement at a subsequent stage in the process. The concentration of undissociated ammonia in the cell closely paralleled that in the external solution over a wide range of pH. The results are in contrast to those of DeTurk (6) with *Pseudomonas* who suggested an active transport mechanism with a simultaneous oxidation requirement.

The stimulatory action of Krebs cycle acids in ammonia utilization, already recorded by numerous workers, was attributed primarily to a buffering effect. Morton & Macmillan (11), using *S. brevicaulis* and physiologically similar fungi, ascribed incomplete assimilation from ammonium sulfate to a fall in the pH of the medium induced by an initial uptake of ammonia. Complete assimilation of ammonia occurred in the presence of 13 organic acids. Apparao's results with *Piricularia oryzae* (16), the fungus causing blast disease of rice, suggested that the ability of fungi to utilize different forms of nitrogen depended on pH changes during growth. He also postulated that the organic acids acted as buffering agents in the medium during growth. Warner (17) was interested in the nitrogen compounds which were used for growth when bacteria were grown in a complex medium offering alternatives for the essential nutrients. The resulting proteins of *Leuconostoc mesenteroides* and a coliform organism grown in a medium containing salts, yeast, and meat extracts, bacteriological peptone and added L-($C^{14}$) lysine, and ($N^{15}$) ammonia showed a very high abundance of $N^{15}$ in the amide nitrogen, a low content in several amino acids, and none in the lysine. The ($C^{14}$) lysine was incorporated at a level of radioactivity lower than that of the lysine in the medium, suggesting that both bacteria utilized peptide in addition to free lysine and ammonia.

The nitrogen requirements of most algae appear to be satisfied by the addition of either $NH_4^+$ or $NO_3^-$ salts to the culture medium. A number of reviews on the subject [Pringsheim (18); Myers (19); Fogg (20)] agree that ammonia is preferentially assimilated, nitrate being used only after the former has been exhausted. Although considerable work has been done on nitrogen assimilation in green algae, most of it has been confined to a few species of *Chlorella, Scenedesmus,* and *Ankistrodesmus.* Of six algae, including four flagellates grown in pure culture containing $NH_4NO_3$, Proctor (21) found that *Haematococcus pluvialis* and *Pandorina morum* prefer-

entially assimilated nitrate in that it was taken up at a faster rate than ammonia. The other algae assimilated ammonium nitrogen primarily. Syrett (22, 23) noted that an increased rate of respiration and a decrease of intracellular carbohydrate accompanied assimilation of ammonia and nitrate by nitrogen-starved cells of *Chlorella vulgaris*. The rate of assimilation of ammonia was four times as fast as that of nitrate. Assimilation of both forms of nitrogen ceased after the same period of time at which point both cultures still contained inorganic nitrogen while the ammonia-treated cells contained considerably less carbohydrate than the nitrate-treated cells. The latter had not recovered from nitrogen starvation since they assimilated added ammonia rapidly, and continued to assimilate nitrate rapidly, if glucose was added. Ammonia assimilation by nitrogen-starved cells of *Chlorella* in darkness was accompanied by more carbon dioxide fixation than that which accompanied nitrate assimilation. Syrett (24) interpreted this as a lower rate of conversion of amino acids from carbohydrate via the tricarboxylic acid cycle in the nitrate-treated cells. The rapid assimilation of small quantities of ammonia, nitrate, or nitrite in *Chlorella* is correlated with high rates of gas exchange which revert back to control values upon depletion of the added nitrogen [Syrett (25)]. A twofold stimulation of oxygen uptake by $NH_4^+$ or $NO_3^-$ added to suspensions of the fungus *Scopulariopsis brevicaulis* in the presence of glucose was noted by Macmillan (26). The stimulatory effect was abolished by 2, 4-dinitrophenol ($2{\cdot}3 \times 10^{-3}M$) although endogenous respiration was enhanced.

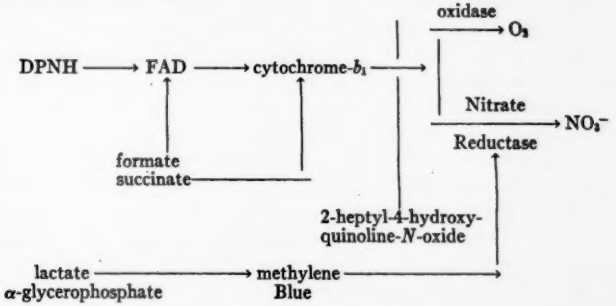
Morton & Broadbent (27) analyzed the organic nitrogen compounds which appeared in the culture medium during ammonia assimilation by *S. brevicaulis, Aspergillus niger,* and other fungi. The formation of extracellular organic nitrogen compounds in the medium during growth on inorganic nitrogen sources is a frequent occurrence in bacteria, blue-green algae, and fungi [references cited by Morton & Broadbent (27)]. They observed that organic nitrogen rose rapidly from the third to the seventh day precisely during the time when ammonia was most rapidly disappearing. The proportion of extracellular nitrogen varied with the fungal species and the nature of the nitrogen source. In *S. brevicaulis* 20 to 25 per cent of the nitrogen was finally found in extracellular form whether the source was ammonia or nitrate. Most of it appeared to be peptide in nature yielding some 14 amino acids on hydrolysis. The fungus was unable to assimilate the peptides but assimilated their constituent amino acids upon their liberation by acid hydrolysis. Dulaney, Bilinski & McConnell (28) obtained somewhat similar results with *Ustilago maydis* grown on an ammonia source in which the bulk of extracellular nitrogen was found as small peptides or free amino acids. Hall & MacVicar (29) observed an extremely rapid and almost complete disappearance of added $N^{15}$-nitrate during the first 45 min. of incubation of *Bacillus subtilis* with a concommitant appearance of highly labeled ammonia in the medium. This was taken as direct exidence that ammonia is an intermediate in nitrate assimilation. Neither hydroxylamine,

oximino nitrogen compounds, hydroxamic acids, nor hyponitrous acid were detected. Burma & Burris (30) reported that cell-free preparations of *Azotobacter vinelandii,* unlike the intact cells, showed no lag in their rapid incorporation of $N^{15}H_4$ into amino acids. The rate of $N^{15}H_4$ uptake was stimulated about fivefold by added α-ketoglutarate and, as in intact cells, glutamic acid had the highest concentration of $N^{15}$ among the amino acids isolated. The results further support the ketoglutarate-glutamate system as the chief means of conversion of ammonia to the amino acid level.

### Nitrate Respiration, Denitrification, and Nitrate Assimilation

Many microorganisms and higher plants absorb and utilize various types of nitrogen compounds including the inorganic salts of nitrate, nitrite, and ammonia with various degrees of efficiency. For a number of microorganisms nitrate is the major source of nitrogen and the biological process by which it is converted to the ammonia or amino acid level for ultimate protein synthesis is known as "nitrate assimilation." Nitrate may also be used by certain microorganisms under anaerobic or partially anaerobic conditions as a terminal electron acceptor in place of oxygen. In many cases, these are presumably energy-yielding reactions and under the given conditions are necessary for the growth and well-being of the organism. This is spoken of as "nitrate respiration" [Sato (31)] or "true-dissimilatory nitrate reduction" [Kluyver (32); Verhoeven (33)]. The former term is preferentially used in the present review. Depending upon the microorganism and the conditions, nitrate may be reduced only to nitrite or, in a series of steps which possibly also provide energy, to more reduced forms of nitrogen including the highest reduction state, ammonia. If molecular nitrogen or, in some cases nitrous oxide ($N_2O$), is the product of nitrate respiration, the process is known as "denitrification." Finally, the situation in which nitrate acts as a hydrogen acceptor in place of other natural substances and thereby interferes with the normal energy-yielding process of the cell, was designated as "incidental dissimilatory nitrate reduction" [Kluyver (32); Verhoeven (33)]. For example, the main effect of nitrate addition to sugar-fermenting bacteria such as *E. coli* and *Clostridium perfringens* was a reduction to nitrite or ammonia accompanied by changes in the nature of the fermentation products. Sato (34) questioned the need for the latter category and, in turn, classified the nitrate-reducing microorganisms into three groups according to the behaviour of their cytochrome systems toward nitrate [Sato (35)]: (*a*) those whose cytochromes participated in nitrate reduction, since their hemoproteins remained at a certain oxidized state during the nitrate-reduction process and resumed their reduced state upon exhaustion of the nitrate. Oxygen was a potent inhibitor. Examples cited are *E. coli* and *Micrococcus denitrificans;* (*b*) those in which the reduced state of the cytochromes is not affected by the nitrate-reduction process, for example, *Neurospora crassa* and *B. subtilis;* and (*c*) those nitrate reducers which lack cytochrome pigments, for example, *Clostridium perfringens.*

*Nitrate respiration in microorganisms which do not carry on denitrification.*—Taniguchi, Sato & Egami (36) formulated the following chain of electron transport involving the reduction of nitrate and oxygen in a particulate preparation of *E. coli:*

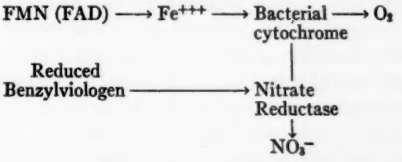



DPNH and FAD were shown to participate. Cytochrome-$b_1$ was also implicated in view of spectral studies and the large inhibition by 2-heptyl-4-hydroxyquinoline-*N*-oxide, a specific inhibitor of the oxidation of cytochromes-*b* and-$b_1$ with formate as the electron donor. In this system nitrate reductase is defined as the terminal member of the electron transport chain reacting directly with nitrate. Earlier, Sato & Egami (37) had suggested that nitrate reductase of *E. coli* was an iron enzyme quite probably identical with cytochrome-*b*. This hypothesis, based on light-reversible enzymatic inhibition by CO, was withdrawn by Sato & Niwa (38) who, nevertheless, believe that *E. coli* nitrate reductase is an iron-containing system. As shown in the above sequence it was consequently concluded that nitrate with the terminal nitrate reductase served as a cellular oxidant in anaerobiosis, instead of oxygen and the terminal respiratory oxidase. The same electron transport chain, except for the terminal steps, therefore, appears to be involved in both the aerobic and the anaerobic states. Since nitrate respiration under anaerobic conditions takes the place of "oxygen respiration," it would be expected that the former would be coupled to the formation of energy-rich compounds. Indirect evidence for the coupling of phosphorylation to nitrate respiration was provided by Takahashi, Taniguchi & Egami (39). They observed that the anaerobic incorporation of $P^{32}$ in resting cells of *E. coli* was enhanced by the addition of nitrate with formate as the electron donor. Asnis, Vely & Glick (40) reported that cytochrome-containing particles of *E. coli* strain ECFS catalyzed the reduction of nitrate by succinate, lactate, formate, α-glycerophosphate, DPNH, or $H_2$. Erkama, Häggman & Wahlroos (41) found that nitrate reduction by $H_2$ or acetate in iron-deficient *E. coli* cells was appreciably suppressed under anaerobic conditions although nitrite reduction was unaffected. When heavy metals were removed by 8-hydroxyquinoline-chloroform extraction no growth oc-

curred under semiaerobic conditions unless separately sterilized Co or Mo was added. Aerobic growth was inhibited despite the presence of Co or Mo. They proposed an electron transport sequence for nitrate reduction essentially similar to that indicated by Egami's group. There is other evidence suggestive of additional or alternate mechanisms of nitrate reduction in the same organism. Nicholas & Nason (42) purified and characterized a DPN-linked nitrate reductase from *E. coli* with FAD and probably Mo as cofactors, thus resembling the corresponding molybdoflavoproteins in *Neurospora* and soybean leaves [Nason & Evans (13); Evans & Nason (43); Nicholas & Nason (44, 45, 46)]. Apparently a nitrate respiration system and a nitrate assimilation system, the latter represented by the molybdoflavo-nitrate reductase, are present in *E. coli* depending upon conditions of growth. Wainwright (47), working with a cell preparation of aerobically grown *E. coli* (strain 1433), observed a pyridine nucleotide-nitrate reductase which, in the presence of FAD or FMN, was stimulated by menadione and ferrous ion. He indicated that cytochrome was possibly a component of the system. Lascelles (48), using a variant strain of *Staphylococcus aureus* which required hematin in order to reduce nitrate to nitrite, observed that the bacterium was capable of nitrate respiration. The organism did not grow under anaerobic conditions either in the presence or absence of hematin unless nitrate was added to the medium. Maximal growth was the same under aerobic conditions and showed a similar hematin requirement. The evidence suggests that the hematin, probably as a cytochrome, is a component of both the nitrate respiration and aerobic respiration systems. This fits in with the above scheme of electron transport proposed by Egami's group. The requirement is somewhat similar to that found in *Haemophilus influenzae* where nitrate conversion to nitrite occurs in cultures grown with hematin or with protoporphyrins, but not in cultures grown with other iron porphyrins which support growth [Granick & Gilder (49)]. The latter workers observed that iron-free porphyrins other than protoporphyrin did not support the growth of a number of smooth strains of *H. influenzae*. These differ from protoporphyrin in the substitution of hydroxyethyl, ethyl, or hydrogen for the vinyl groups at positions 2 and 4. The vinyl groups are not important for growth if an iron-containing porphyrin is supplied, and may be either directly or indirectly concerned with the insertion of iron into the protoporphyrin ring. The vinyl groups are also needed in the function or formation of the system responsible for the conversion of nitrate to nitrite by *H. influenzae*. Granick & Gilder (49) showed that in the presence of iron protoporphyrin, or protoporphyrin, nitrate reduction was proportional to growth. When growth was supported by other iron porphyrins, nitrite formation did not occur. A competition existed between iron protoporphyrin and iron mesoporphyrin (ethyl groups substituted for the vinyl groups) in nitrate reduction, the process decreasing as the concentration of iron mesoporphyrin was increased. The work of Granick & Gilder offers a basis for the present-day evidence implicating

cytochromes in bacterial nitrate-reducing systems. In addition, it provides evidence concerning the structural requirements for nitrate reduction, namely, that vinyl groups on the porphyrin portion of the hemoprotein are necessary for conversion of nitrate to nitrite. Perhaps the vinyl groups directly or indirectly affect attachment to a particular apoenzyme or influence the specificity of the latter, thus conferring the property of nitrate reduction.

Sadana & McElroy (50) purified and characterized a nitrate-reducing system from the salt-water luminous bacterium, *Achromobacter fischeri*, and proposed the following pathway of electron transport:




They separated the electron transport chain into two soluble portions, (*a*) the electron-donor system, namely DPNH-cytochrome-*c* reductase with a requirement for FMN or FAD, and (*b*) the terminal system, nitrate reductase, by which electrons are transported from the reduced cytochrome to nitrate. The cytochrome component which is autoxidizable has absorption bands similar to mammalian cytochrome-*c*. Although the nitrate reductase was not prepared free of bacterial cytochrome, they feel that the enzyme can catalyze the reduction of nitrate using reduced cytochrome. When reduced benzylviologen supplied electrons, the bacterial cytochrome was not involved as indicated by spectral studies and the lack of inhibition by CO (and other metal-binding agents) in contrast to the photoreversible inhibition by this reagent with DPNH as the electron donor. Further evidence favoring cytochrome and iron as essential components of the nitrate-reducing system was provided by nutritional and inhibitor studies. Although the proposed electron transport chain is similar in some respects to that suggested for *E. coli* by Taniguchi, Sato & Egami (36), it indicates still another variation of a nitrate-reducing pathway. In view of the cytochrome involvement and the apparent competition by $O_2$ for electrons in the conversion of nitrate to nitrite, the nitrate-reducing system of *A. fischeri* is tentatively classified under nitrate respiration.

*Nitrate respiration in denitrifying organisms.*—The first clear knowledge of denitrification stems from the classic work of Gayon & Dupetit (51). They introduced the term for the conversion of nitrate to molecular nitrogen or to nitrous oxide, and showed that it resulted from the activity of bacteria which substitute nitrate for oxygen in the oxidation of organic substrates. These facultative organisms, which have since been found almost universally in soil and water, utilize nitrate as a hydrogen acceptor for energy-yielding oxidative reactions. The articles by Kluyver (32), Ver-

hoeven (33), Delwiche (52), Verhoeven & Takeda (53), and Najjar & Chung (54) collectively cover the field of denitrification until 1955. Sreenivasan & Venkataraman (55) identified 20 of the 32 denitrifying isolates obtained from the sea water off the South Indian coast as belonging to the *Pseudomonas*. They suggested that the apparent nitrate deficiency in the waters of tropical seas [Harvey (56)] was possibly due to denitrification. Hydroxylamine was toxic to all the isolates. A minimum amount of oxidizable organic matter, probably provided by plankton, was required. They indicated, on the basis of the literature, that the known denitrifying bacteria can be classified into three genera: *Micrococcus, Denitrobacillus,* and *Pseudomonas*. This is an oversimplification since there are a wide variety and large number of denitrifying microorganisms which include species of *Spirillum, Bacillus, Achromobacter,* and some autotrophs such as *Thiobacillus denitrificans* and *T. thioparus* [see Delwiche (52)].

*Electron source.*—For most denitrifyers, organic compounds serve as the energy source with either oxygen or nitrate as the ultimate electron acceptor. In the particular case of *Micrococcus denitrificans* it was demonstrated by Kluyver & Verhoeven (57) that molecular hydrogen or organic substrates can be used as a hydrogen donor in nitrate reduction to gaseous nitrogen and nitrous oxide. This bacterium is regarded as a heterotroph. To some extent it can also be considered a facultative autotroph, although certain organic growth substances are essential [Kluyver (32)]. The demonstration of denitrification with the concomitant oxidation of sulfur [Beijerinck (58)] or thiosulfate [Lieske (59)] by *Thiobacillus denitrificans* appears to be at the expense of sulfur as the electron donor. Sacks & Barker (60) and Verhoeven & Goos (61) previously demonstrated, for some bacteria, that a number of organic substrates in the denitrification process are quantitatively converted into carbon dioxide. It has long been known that denitrifying bacteria are unable to utilize aromatic acids as substrates anaerobically in the presence of nitrate. Hansen & Kallio (62) added a second exception to the generalization that all compounds which serve aerobically as energy sources for denitrifying or nitrate-reducing bacteria serve in the same capacity anaerobically with nitrate as the hydrogen acceptor. They were unable to observe anaerobic oxidation of various straight-chain aliphatic hydrocarbons in the presence of nitrate by *Pseudomonas stutzeri,* although the hydrocarbons were readily oxidized aerobically. The homologous oxygen-containing compounds, however, were readily oxidized anaerobically. The suggestion by Stanier (63) that aromatic acids cannot function as oxidizable substrates for denitrifying organisms anaerobically in view of the need for molecular oxygen for the primary attack on the aromatic nucleus by introduction of hydroxyl groups (oxygenation), was extended to the hydrocarbons by Hansen & Kallio. They indicated that the primary enzymatic attack on the hydrocarbon molecule may involve direct participation of molecular oxygen as in the case of pyrocatechase [Hayai-

shi, Katagiri & Rothberg (64)]. The evidence offered by Hansen & Kallio supports the suggestion that oxygenation is probably the first step in the biological oxidation of hydrocarbons and that anaerobic oxidation of hydrocarbons, with the possible exception of methane, by nitrate-reducing or sulfate-reducing bacteria is an infrequent occurrence.

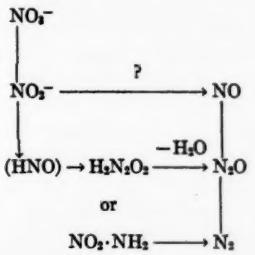
*Oxygen.*—The quantitative effect of oxygen on denitrification has been the subject of numerous conflicting reports. The general view is that oxygen is a potent inhibitor of the denitrification process by virtue of its effective competition with nitrate as an acceptor of electrons in the oxidative functions of the cell [Delwiche (52)]. Sacks & Barker (65), Wijler & Delwiche (66), and others showed that low oxygen tensions (approximately one to four per cent) suppressed denitrification as much as 90 per cent. Of some interest is Verhoeven's observation (67) with *Denitrobacillus licheniformis* that while molecular nitrogen and nitrous oxide are normal anaerobic denitrification products and vigorous aeration largely prevented nitrate reduction, small amounts of oxygen resulted in the conversion of a large percentage of nitrite to ammonia. Skerman & MacRae (68) observed that no nitrate reduction by *Pseudomonas denitrificans* occurred when oxygen was continually present in the surrounding fluid even at low concentrations. They concluded that the competition between oxygen and nitrate for donor electrons so favored the former that nitrate reduction occurred only when the supply of oxygen was inadequate to meet the demands. Nitrate reduction occurred when the oxygen concentration was below the critical level at which oxygen-utilizing systems were saturated; and that when it occurred in aerated cell suspensions it was caused by the activity of cells deprived of the oxygen supply. Collins (69), working with *Pseudomonas aeruginosa,* and Fedorov & Sergeeva (70), working with *P. fluorescens* and *P. pyocyanea,* also reported the phenomenon of aerobic denitrification and attributed it to a failure to meet the high oxygen requirements of the rapidly multiplying aerobic organism in liquid media. Many methods of forced aeration in deep cultures are insufficient to maintain oxygen saturation, especially when growth becomes dense. The latter workers ascribed this in part to the slow diffusion of oxygen into the medium. By lowering or raising the partial pressure of oxygen in the medium they inversely affected the reduction of nitrate. Kefauver & Allison (71) observed that *Bacillus denitrificans* (presumably *Pseudomonas stutzeri*) was capable of using simultaneously both molecular oxygen and nitrite.

*Cytochromes.*—The typical pink color of denitrifying bacteria when grown under anerobic conditions in the presence of nitrate was first reported by Sacks (72), noted also by Allen & Van Niel (73), and Baalsrud & Baalsrud (74), and attributed to bacterial cytochrome. Sacks (72), using cell-free extracts of *Pseudomonas denitrificans,* observed absorption peaks at 552 mμ and 522 mμ indicative of the α- and β-bands of a cytochrome-*"c."* In intact cell suspensions these peaks disappeared upon aeration and ap-

peared when oxygen was removed. The addition of nitrate or nitrite anaerobically also caused the bands to fade, thus implicating cytochrome in the reduction of these nitrogen compounds. The bacterium, when grown anaerobically in a nitrate-glucose medium, contained much more of this cytochrome-*c*-like pigment than when grown aerobically. Kamen & Vernon (75) noted that a number of photosynthetic bacteria and two denitrifyers, *Micrococcus denitrificans* and *Pseudomonas denitrificans*, contained cytochromes of the "*c*" type. The isolated cytochrome from *M. denitrificans* showed spectroscopic and electrochemical properties identical with those of mammalian cytochrome-*c*, whereas the cytochrome from *P. denitrificans* resembled that of the photosynthetic bacteria. In *M. denitrificans* the yield of cytochrome-*c* was not appreciably affected by anaerobic or aerobic conditions. The existence of a "*c*" type cytochrome in the anaerobic bacterium *Desulfovibrio desulfuricans* and its subsequent oxidation upon addition of sulfate or sulfite to a cell suspension was already noted by Postgate (76). Vernon (77) further examined the cytochromes of *M. denitrificans* and *P. denitrificans* and found that in addition to the "*c*" type, both organisms contained a soluable "$b_1$-" type cytochrome with absorption maxima at 559, 528, and 426 mμ. Reduced cytochrome-$b_1$ was rapidly oxidized by nitrate with intact cells and purified enzyme fractions. Adding nitrate to a purified sample of reduced *P. denitrificans* cytochrome-*c* gave no oxidation of the cytochrome after a few minutes, whereas cytochrome-$b_1$ was immediately oxidized. He therefore attributed Sachs' observations of cytochrome-"*c*" oxidation in intact cells by nitrate to a secondary effect. Vernon (78) also found three soluble cytochromes, apparently *c*, $c_1$, and $b_1$ in an unidentified pseudomonad capable of reducing nitrate. Lenhoff, Nicholas & Kaplan (79) obtained data suggesting that the oxygen tension of the medium during the growth of a *Pseudomonas fluorescens* strain determined the alternative routes of terminal electron transfer formed by the organism. Extracts from cells grown without aeration in a nitrate-containing medium had large amounts of cytochrome pigment, cytochrome peroxidase and catalase, and moderate diaphorase activity. In contrast, extracts of cells grown with aeration had only a trace of cytochrome pigment and cytochrome peroxidase, slight catalase activity, and greater diaphorase activity. The cytochrome-rich cells grown at low oxygen tension showed a higher iron requirement and a lower molybdenum requirement than those grown at high oxygen tension. Under the latter conditions, the molybdenum content was five times that of cells grown at low oxygen tensions. They concluded that the iron-cytochrome peroxidase pathway prevailed in cells grown at low oxygen tension, whereas the molybdenum flavoprotein pathway was the terminal oxidase system for cells grown at high oxygen tension. The increase in specific activity of nitrite reductase at low oxygen tensions on an iron-deficient medium concomitant with only a trace of cytochrome-*c* in the cells indicated to them that cytochrome-*c* was not involved in nitrate and nitrite reduction. Our present knowledge of bacterial cytochromes in nitrate respiration suggests as an

alternative interpretation that nitrate is the terminal electron acceptor under anaerobic conditions. Presumably, the affinity of the terminal cytochrome system for nitrate is considerably less than for oxygen, with the organism responding to anaerobic conditions by producing larger concentrations of cytochrome. In the aerobically-grown cells the molybdoflavoprotein serves primarily in nitrate assimilation, whereas anaerobically the cytochrome-nitrate system functions as the terminal respiratory step.

*Denitrification pathways.*—Kluyver (32), Verhoeven (33), and Delwiche (52) summarized the biochemical pathway for denitrification essentially as follows (the sequence for reduction to ammonia is considered in a later section):




The above scheme postulates two-electron steps. The first step involves the conversion of nitrate to nitrite. A number of enzymatic mechanisms for the reduction of nitrate to nitrite were already established for different organisms including the *Neurospora* molybdoflavoprotein pathway [Nason (80)] and the cytochrome system of *E. coli* [Taniguchi, Sato & Egami (36)]. The enzymatic mechanism for a denitrifying organism was in part elucidated by Najjar & Chung (54) and by Chung & Najjar (81), and is discussed below. The next step, the reduction of nitrite is not clearly understood and on the basis of a presumed two-electron change would result in formation of the hypothetical nitroxyl intermediate (perhaps as a nitroxyl-enzyme combination). The presumed spontaneous dimerization of nitroxyl to yield hyponitrite (or nitramide) might be followed by a spontaneous or enzymatic decomposition of hyponitrite involving the splitting off of water thereby yielding nitrous oxide ($N_2O$). The enzymatic reduction of the latter to molecular nitrogen was demonstrated in a cell-free system [Chung & Najjar (81)]. Alternatively, the enzymatic hydrogenation of hyponitrite (or nitramide) might lead directly to the formation of nitrogen gas. The claim by Allen & Van Niel (82) that *Pseudomonas stutzeri* hydrogenated nitramide to gaseous nitrogen was refuted by Kluyver & Verhoeven (83) who found that nitramide was decomposed too rapidly upon addition of phosphate buffer to be used as a substrate. The latter workers showed that neither *Micrococcus denitrificans* nor *Pseudomonas aeruginosa* evolved nitrogen from sodium hyponitrite, confirming the results of Allen & Van Niel (82)

with *P. stutzeri*. Sacks & Barker (60) concluded that nitrous oxide was not an obligatory intermediate in the formation of molecular nitrogen since (*a*) they could not detect nitrous oxide in their denitrification experiments with *Pseudomonas denitrificans;* (*b*) the utilization of nitrous oxide was selectively blocked by azide or dinitrophenol under conditions which permitted the formation of nitrogen from nitrite; and (*c*) a lag frequently preceded the utilization of nitrous oxide by resting cells, a phenomenon not observed in the conversion of nitrite to nitrogen. Allen & Van Niel (82) also observed that the conversion in *P. stutzeri* of nitrous oxide to nitrogen was inhibited by cyanide, whereas the reduction of nitrite to nitrogen was unaffected. They reported a simultaneous adaptation to nitrous oxide by cells grown on nitrate as did Kluyver & Verhoeven (83) with *M. denitrificans* and *P. aeruginosa*. Nitric oxide (NO) was recently implicated in denitrification with cell-free bacterial preparations by Najjar and his co-workers (54, 81) and with mixed denitrifying cultures by Wijler & Delwiche (66). The first demonstration at the cell-free level of the reduction of nitrate or nitrite to molecular nitrogen, nitrous oxide, and nitric oxide was made by Najjar & Allen (84) with preparations from *P. stutzeri* and *Bacillus subtilis*. Chung & Najjar (54, 81) subsequently showed that crude ammonium sulfate fractions of *P. stutzeri* extracts catalyzed the formation of nitric oxide and molecular nitrogen from nitrite. In the reduction of nitrite to nitric oxide TPNH and DPNH served as electron donors, FAD or FMN gave a twofold stimulation, and a number of metal-binding agents significantly inhibited the enzyme. Nitric oxide reductase activity ($NO \rightarrow N_2$) in similar fractions likewise exhibited a pyridine nucleotide requirement, a stimulation by FAD or FMN and an even greater sensitivity to metal-binding agents than the nitrite reductase. The partial loss in activity of both enzymes after successive dialysis against solutions of metal-chelating agents and water was restored to a small extent by the addition of $Cu^{++}$, $Fe^{++}$, or $Fe^{+++}$ salts. An examination of the effect of these metals at the nonenzymatic level would be pertinent since $Cu^{++}$ was noted to catalyze the nonenzymatic disappearance of nitrite [Spencer, Takahashi & Nason (85)]. The crude enzyme fractions studied by Chung & Najjar contained substantial amounts of cytochrome-*c* which could not be readily separated. The following was suggested:

$$\begin{array}{lllll} & & & & NO_2^- \\ & & & & \nearrow \\ \text{TPNH} \rightarrow & \text{FAD} \rightarrow & Cu^{++} \rightarrow & \text{cytochrome} & \\ \text{or} & \text{or} & \text{or} & & \searrow \\ \text{DPNH} & \text{FMN} & Fe^{+++} & & NO \end{array}$$

In view of the limited data on identification of the metal component, the inclusion of copper or iron in this scheme must be regarded as tentative. Yamada & Virtanen (86) found that a halotolerant strain of *Micrococcus* as a cell suspension, or a cell-free preparation, reduced nitrite (or nitrate) to nitrous oxide with practically no conversion to molecular nitrogen,

hydroxylamine, or ammonia. Methylene blue was used as an intermediate hydrogen carrier. Iwasaki, Matsubayashi & Mori (87) and Iwasaki & Mori (88) reported that nitric oxide was produced from nitrite by denitrifying bacteria with a dye as a hydrogen carrier. A cyanide-sensitive soluble enzyme catalyzed the conversion of nitrite to nitrogen using added dye with nitric oxide postulated as an intermediate.

*Adaptation.*—Earlier studies, particularly by Pollock (89), with nitrate-respiring organisms including the denitrifyers, demonstrated the adaptive nature of the nitrate-reducing enzyme system [see reviews by Kluyver (32) and Delwiche (52)]. This was indicated by a lag period in the reduction of nitrate by cells which had not originated in a nitrate-containing medium. Aerobically-grown cells experienced a lag in nitrite utilization whereas no lag in oxygen utilization occurred with anaerobically-grown cells. Wainwright & Nevill (14), working with nitrogen-depleted *E. coli,* observed a rapid and marked increase in nitrate reductase activity (with formate as the hydrogen donor), the maximal value being reached within 2 hr. after adding nitrate. There was also little or no lag in the formation of tetrathionate reductase, whereas an appreciable delay occurred in the appearance of induced β-galactosidase activity in the presence of exogenous nitrogen and lactose. The marked stimulation by added casein hydrolysate (but not by ammonium sulfate) of induced biosynthesis of nitrate reductase by nitrogen-depleted cells to added nitrate suggested that the limiting factor was a limiting rate of *de novo* synthesis of amino acids. Wainwright & Nevill (90) also showed that auxotrophic mutants of *E. coli* requiring tryptophan, arginine, histidine, or proline did not grow or form nitrate reductase in the absence of the appropriate amino acid. Cells of a uracil-requiring strain did not grow and formed little nitrate reductase in the absence of uracil in comparison to that obtained when uracil was present. A thymine-requiring strain, however, formed nitrate reductase as readily in the absence or presence of thymine. The results with the mutants requiring uracil or thymine suggest that ribonucleic acid synthesis, which is required for protein synthesis, is necessary for the synthesis of the nitrate-reducing system. Straughn (91) obtained highly active preparations of nitrite reductase from *E. coli,* Crookes strain, grown in a one per cent peptone broth with vigorous aeration. He concluded that the enzyme was not adaptive under these conditions since the addition of nitrate to the growth medium or other attempts to adapt cell suspensions failed to enhance nitrate reductase activity. Aside from the usually invoked argument of strain difference, there is a possibility that nitrate was formed in the medium by the oxidation of peptone under the growth conditions of vigorous aeration with subsequent enzyme adaptation. Farkas-Himsley & Artman (7) reported that nonproliferating suspensions of old *E. coli* cells grown on a peptone medium supplemented with nitrate, reduced nitrate at a higher rate and without a lag period as compared to similar cells from a nitrate-less medium. The cell-free extracts, however, reduced nitrate at the same rate

whether derived from cells grown with or without nitrate. Nonproliferating young cells reduced nitrate at the same high rate regardless of the presence or absence of nitrate in the growth medium. While the kinetics of nitrate reduction by nonproliferating suspensions of old cells in medium with and without nitrate suggested an adaptive system, the high rate of nitrate reduction by their respective cell extracts indicated instead a permeability barrier in old cells toward nitrate. Peptone was regarded as a stabilizer of the system. The possibility exists that although nitrate reductase is in reality a constitutive system, the system concerned with nitrate transport may be the adaptive one. The molybdoflavoprotein system of *Neurospora,* however, is truly adaptive since cell-free preparations of mycelia grown on ammonium salts or amino acids showed little or no nitrate reductase activity in comparison to the high enzymatic activity in nitrate-grown mats [Nason & Evans (13)].

*Future problems in denitrification.*—Although a start has been made in enzyme characterization, a good deal of work remains. Of special interest would be the demonstration of the presumed coupling of phosphate esterification with the process of nitrate respiration, particularly in denitrification. If oxidative phosphorylation occurs as suggested by the results of Takahashi, Taniguchi & Egami (39) does it take place in the step involving the reduction of nitrate, or will phosphate be esterified into high-energy bonds when nitrogen compounds or intermediate oxidation states, such as nitrite or hydroxylamine, act as electron acceptors?

## Nitrate Assimilation

*Reduction of nitrate to nitrite.*—The first step in nitrate assimilation is catalyzed by nitrate reductase. The enzyme was characterized from *Neurospora* and soybean leaves by Nason & Evans (13) and by Evans and Nason (43) as a sulfhydryl metallo-FAD-protein. *Neurospora* nitrate reductase is relatively specific for TPNH as the electron donor, while TPNH and DPNH are equally effective with the soybean enzyme. That molybdenum is the metal constituent of the *Neurospora* enzyme [Nicholas & Nason (44)] was shown by the (*a*) increased nitrate reductase activity in various protein fractions which was accompanied by increased molybdenum concentration; (*b*) decrease in molybdenum concomitant with a decrease in enzyme activity during dialysis against cyanide; (*c*) specific reactivation of the cyanide-dialyzed enzyme by molybdenum; and (*d*) specific effect of molybdenum deficiency during growth resulting in decreased nitrate reductase activity. Nicholas & Nason (45) demonstrated that flavin and molybdenum function as electron carriers in *Neurospora* nitrate reductase in the following sequence:

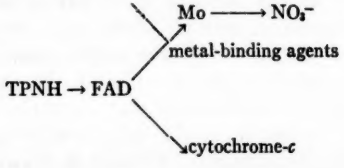
$$\text{TPNH} \rightarrow \text{FAD (or FMN)} \rightarrow \text{Mo} \rightarrow \text{NO}_3^-$$

Flavin precedes molybdenum in the above scheme as shown by the inability of the molybdenum-free enzyme to catalyze the reduction of nitrate to nitrite by TPNH or reduced flavin, although FAD or FMN was reduced by

TPNH. Added molybdenum specifically restored the ability of the enzyme to catalyze nitrite formation by reduced flavin or TPNH plus flavin. That molybdenum functioned as electron carrier was strongly suggested by experiments in which reduced molybdate prepared with sodium hydrosulfite enzymatically reduced nitrate to nitrite. The electron transport role was supported by the observation that molybdate enzymatically oxidized $FMNH_2$. Final proof for the oxidation-reduction role of molybdenum in nitrate reductase, however, must await direct evidence with the endogenous molybdenum in the intact enzyme. Similar studies identifying molybdenum as the metal component and demonstrating the sequence and mechanism of electron transport to be the same as in *Neurospora* were made with the nitrate reductase from soybean leaves [Nicholas & Nason (46)]. That molybdenum is a component of soybean leaf nitrate reductase was subsequently confirmed by Evans & Hall (92). They ascribed the small reactivation of the dialyzed enzyme by iron to partial replacement of molybdenum without discussing the possibility of a molybdenum contamination in the iron solution. A pyridine nucleotide-nitrate reductase in *E. coli* and its identification as a metalloflavoprotein with molybdenum as the probable metal component [Nicholas & Nason (42)] was already mentioned in relation to the nitrate respiratory system in this bacterium (see page 210). Evans (93) and Cheniae & Evans (94, 95) claimed a relation between nitrogen fixation by soybean nodules and the nitrate reductase of *Rhizobium japonicum* in the nodules. The enzyme is a DPN-specific system having a metal component and a probable flavin requirement. They correlated the nitrogen-fixing capacity of the soybean plant, the nitrate reductase of the bacterial suspensions from the nodules, and the hemoglobin content of the nodules. Their suggestion that chemisorbed nitrogen or an oxidized product induced adaptive formation of nitrate reductase in the nodule bacteria was criticized in a recent review by Nicholas (96) on the grounds that it was difficult to eliminate nitrate-N completely from salts used in culture solutions. Cheniae & Evans had reported, however, that nodule nitrate reductase was not adaptive to externally supplied nitrate although *R. japonicum* cultures grown on liquid media were, thus implying the existence of a permeability barrier in the nodules to exogenous nitrate. The possibility of incidental nitrate formation within the nodule by oxidation of a product of nitrogen fixation, perhaps by a peroxidase reaction, must also be considered since traces of nitrate are sufficient for adaptive formation of nitrate reductase.

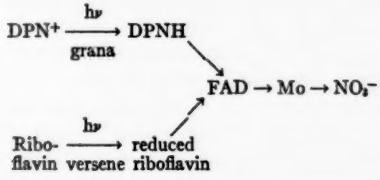
Nicholas & Stevens (97) reported the isolation and separation of three oxidation states of molybdenum ($Mo^{+6}$, $Mo^{+5}$, and $Mo^{+3}$) and presented evidence that $Mo^{+5}$ was as effective as TPNH as an electron donor for the enzymatic reduction of nitrate. Their experiments support the suggestion [Nicholas & Nason (45)] that molybdenum enzymatically undergoes a reversible oxidation-reduction beween the plus five and plus six oxidation states. McElroy (98) noted that inorganic phosphate stimulated *Neurospora* nitrate reductase in the molybdenum-requiring step, namely $FADH_2 \rightarrow Mo \rightarrow NO_3^-$. Subsequently, Nicholas & Scawin (99) reported the

same effect by phosphate, selenate, tellurate or arsenate, and, to a lesser extent, by sulfate or silicate. In a detailed paper, Kinsky & McElroy (100) observed that arsenate and tungstate but not silicate could replace the phosphate requirement. They postulated the existence of a phosphomolybdo-complex in nitrate reductase with $FADH_2$ and $FMNH_2$ as specific electron donors. This is analagous to the situation whereby specific reagents are known to reduce phosphomolybdenum complexes but not free molybdate. Thus, the phosphomolybdo-, the arsenomolybdo-, and tungstomolybdo-complexes presumably have oxidation-reduction potentials, activation energies, or steric requirements different from the molybdate ion which facilitate an enzymatic reaction with reduced flavin nucleotides. They also observed that nitrate reductase preparations contained an active TPN-cytochrome-*c* reductase, the ratio of the two activities remaining constant over a wide range of purification and heat inactivation. The similarity of Km values of TPNH and FAD for nitrate reductase and cytochrome-*c* reductase suggested that the two activities were catalyzed by the same enzyme. However, various metal-binding agents were potent inhibitors of nitrate reductase, whereas cytochrome-*c* reductase was unaffected. Adaptation experiments showed that both nitrate and cytochrome-*c* reductase activities were induced in parallel fashion when *Neurospora* was grown in a medium containing varying amounts of nitrate. However, there was no nitrate reductase in mycelia grown on ammonia as the sole nitrogen source, while there was substantial TPN-cytochrome-*c* reductase having approximately 50 per cent of the specific activity of that obtained from nitrate-grown *Neurospora*. Their results point to two kinds of TPN-cytochrome-*c* reductases: (*a*) a constitutive enzyme with no associated nitrate reductase as indicated by the adaptive experiments with ammonia-grown mycelia and (*b*) an enzyme associated with nitrate reductase activity. The data of Kinsky & McElroy suggest that the latter enzyme may be a single system which is responsible for both nitrate and, in part, cytochrome-*c* reduction, or that the two activities possess a common intermediate step, namely the presumably rate-limiting reaction, TPN-flavin reductase. The next step would involve the enzymatic transfer of electrons either to nitrate by way of molybdenum thus giving nitrate reductase activity, or to cytochrome-*c* to give cytochrome-*c* reductase activity. The parallel inducement of both activities in nitrate-grown mycelia would arise primarily from adaptive formation of nitrate reductase, the first step of which (TPNH→Flavin) could also be used by the cytochrome-*c* reductase, thus reflecting itself in a greater cytochrome-*c* reductase activity. This is represented as follows:



The hypothesis implies that at least two component enzymes are involved in the nitrate reductase system although as yet they have not been separated. The same would also apply to the TPN-cytochrome-*c* reductase. A possible explanation for the conclusion of Nason & Evans (13), based on changing activity ratios, that nitrate reductase does not catalyze cytochrome-*c* reduction is that the above two cytochrome-*c* reductases are present in *Neurospora* extracts during the early stages of purification.

Evans & Nason (43), by the use of grana and purified nitrate reductase from soybean leaves, demonstrated the photochemical reduction of nitrate. The observation by Stoy (101) of a significant increase in nitrate reduction by detached wheat leaves in the blue and violet parts of the spectrum suggested involvement of a yellow pigment. He had already shown that photosynthesis and nitrate assimilation paralleled each other in red and green light. In testing the possible role of riboflavin as a light-absorbing catalyst in biological photoreduction, Stoy (102) observed that photoreduced riboflavin in the presence of purified nitrate reductase from wheat leaves served as a more efficient electron donor than DPNH. Versene was required for the riboflavin-coupled system and could not be replaced by cysteine. He proposed the following scheme:




No direct light activation of the flavin-containing nitrate reductase was observed. While the scheme accounts for the observed additional nitrate-reducing effect of blue and violet light, the existence of the photoactivated riboflavin phenomenon and its coupling to the enzyme in wheat leaves remains to be proven. Egami *et al.* (103) reported the presence of a soluble DPN-nitrate reductase and cytochrome-*b* in a bean cotyledon macerate and another more active nitrate reductase in the seedling macerate. The former system was characterized as a sulfhydryl metalloprotein and postulated to participate with cytochrome-*b* in energy-yielding reactions of the nitrate respiration type. The nitrate reductase enzyme in the seedling was regarded as functioning in nitrate assimilation. Purification and further characterization of these systems would be desirable. In contrast to the adaptive nature of nitrate reductase in *Neurospora,* Morton & Macmillan (11) reported that the fungus *Scopulariopsis brevicaulis* contained a nitrate-reducing system which was developed either fully or to a considerable extent in the complete absence of nitrate. Candela, Fisher & Hewitt (104) and Tang & Hsiang (105) recently presented the first evidence for the adaptive formation of a nitrate-reducing system in higher plants as measured with a cell-

free preparation. Silver (106) observed a pyridine nucleotide-nitrate reductase in the extracts of the yeast *Hansenula anomala* grown on a nitrate-containing medium. The enzyme was similar to that found in *Neurospora* and soybean leaves, having a metalloflavoprotein with FAD and molybdenum as probable prosthetic groups. Spectrophotometric observations eliminated a direct role of the cytochromes in nitrate reduction. Costilow & Humphreys (107) found that two strains of *Lactobacillus plantarum* required oxygen for nitrate reduction. They suggested that the family Lactobacteriaceae and the genus *Lactobacillus* as now given in Bergey's *Manual of Determinative Bacteriology* be changed to permit inclusion of certain species which reduce nitrates. While no explanation was given for the oxygen requirement, it seems likly that it was necessary for the formation of the enzyme, probably of molybdoflavoprotein type.

Supporting evidence for the role of molybdenum in nitrate assimilation in the intact organism continues to accumulate. Wolfe (108, 109) observed that molybdenum was required for the healthy growth of the blue-green alga *Anabena cylindrica* on nitrate or molecular nitrogen, whereas it had no effect when ammonium chloride was the sole nitrogen source. However, molybdenum-deficient, nitrogen-starved cells rapidly reduced nitrate to ammonia and amide, but further synthesis to peptides and proteins proceeded very slowly. He ascribed this to an insufficient supply of hydrogen or energy donors in view of the stimulation by succinate and fumarate of nitrate assimilation in molybdenum-deficient cells. The results are puzzling since it is difficult to reconcile his report of complete assimilation of ammonia by molybdenum-deficient cells with his observation that nitrate is rapidly reduced in these cells as far as ammonia and amide only. Arnon *et al.* (110) and Ichioka & Arnon (111) showed that molybdenum was essential for growth and cell division of the green alga *Scenedesmus obliquus* with nitrate as the sole nitrogen source. The metal was not required for growth, nitrogen assimilation, or chlorophyll formation when nitrate was replaced by either urea or ammonium carbonate. Higgins, Richert & Westerfeld (112) demonstrated that tungstate, a dietary antagonist of molybdate in animal nutrition, was also a competitive inhibitor of molybdate in *Aspergillus niger* when nitrate was the sole nitrogen source. A similar type of competitive inhibition of tungstate with molybdate in nitrate assimilation (and nitrogen fixation) by *Azotobacter* was shown by Takahashi & Nason (113). Tungstate itself had no effect on the purified nitrate reductase from *Neurospora*. Candela, Fisher & Hewitt (104) found that the infiltration of molybdenum into leaves of molybdenum-deficient cauliflower plants resulted in an increased nitrate reductase activity in 18 to 24 hr. The enzyme was high in normal plants given nitrate and nitrite, and low, but not negligible, in those provided with ammonium sulfate. Nitrate reductase and nitrite reductase were among the enzymes which underwent a decrease in the temperature-sensitive riboflavin-requiring mutant of *Neurospora crassa* when grown on a riboflavin-deficient medium, while peroxidase and cytochrome

oxidase experienced an increase [Nicholas (114)]. Other recent papers dealing with nitrate reduction include those by Omura (115), who reported the inhibition by acetaldehyde oxime, pyruvic acid oxime, and hydroxylamine of crude nitrate reductase preparations from silkworm, fungi, and fowl. Hattori (116) could not detect urease (or arginase) in *Chlorella ellipsoidea* cells which rapidly assimilated urea, arginine, ammonia, and nitrate. The condition of "oat hay" poisoning in cattle is indirectly due to nitrate which may reach a level of seven per cent (as $KNO_3$) in oat straw [Delwiche (52); Barnett & Brown (117)]. Nitrate is reduced to nitrite by microbial action in the rumen with a resultant toxicity manifested partly by methaemoglobinuria. Nitrite formation from nitrate in the rumen was most vigorous when dried grass or grass pigments were present, whereas no nitrite was detectable in the rumen containing glucose although the nitrate disappeared [Barnett & Brown (117)].

*Nitrite reduction.*—With the elucidation of the enzymatic mechanisms of conversion of nitrate to nitrite, increasing attention has been given to the reduction of nitrite. Yamagata (118) was the first to demonstrate the reduction of nitrite by a cell-free preparation using *Bacillus pyocyaneus.* Taniguchi, Sato & Egami (36), in reviewing their work on the conversion of nitrite and hydroxylamine to ammonia in the presence of *Bacillus pumilus,* reported that methylene blue or $FADH_2$ but not DPNH served as an electron donor. The detection of pyridine nucleotide-nitrite reductase in extracts of *Neurospora* and soybean leaves [Nason & Evans (13) and Evans & Nason (43)] subsequently led to the purification and characterization of pyridine nucleotide-hydroxylamine and nitrite reductases from both sources [Zucker & Nason (119); Nason, Abraham & Averbach (120)]. Both enzymes in *Neurospora* were shown to be metalloflavoproteins with unidentified metal components, whereas the same systems from soybean leaves had a specific requirement for $Mn^{++}$ as measured by both DPNH oxidation and ammonia production. It was indicated [Kluyver (32); Verhoeven (33); Nason (80)] that if two-electron changes are involved in nitrite reduction, then the following sequence with an unidentified intermediate having a nitrogen atom of oxidation number plus one (possibly HNO), $NO_2 \cdot NH_2$ or $H_2N_2O_2$, could be postulated.

$$\underset{+3}{NO_2^-} \rightarrow (HNO),\ \underset{+1}{NO_2 \cdot NH_2},\ \text{or}\ H_2N_2O_2 \rightarrow \underset{-1}{NH_2OH} \rightarrow \underset{-3}{NH_3}$$

Taniguchi, Sato & Egami (36) pointed out that hyponitrous acid was not reduced by a crude extract of a halotolerant bacterium containing all the enzyme systems for reduction of nitrate to ammonia using reduced methylene blue as a hydrogen donor. Evidence for the possible involvement of hyponitrous acid or its isomers in denitrification was treated in a previous section. The report by Medina & Nicholas (121) of a hyponitrite reductase in *Neurospora* is discussed in a later section.

According to Taniguchi, Sato & Egami (36), although *E. coli* was unable

to reduce nitrate beyond the nitrite level under anaerobic conditions, it reduced nitrate to nitrite, hydroxylamine, and ammonia in well-aerated cultures with glucose as the respiratory substrate. McNall & Atkinson (122) state that most strains of *E. coli* reduce nitrate to nitrite at the expense of hydrogen gas, and that there are strains which use glucose to reduce nitrate completely to ammonia. They isolated *E. coli* strain Bn which grows on nitrate or nitrite under molecular hydrogen. It was derived from a parental strain to which nitrate and nitrite were toxic. *E. coli* Bn reduced nitrate completely to ammonia at the expense of hydrogen, whereas the hydrogen uptake of the parental culture corresponded to the reduction of nitrite only. Strain Bn was much more tolerant to nitric oxide than the parental type, apparently because the oxide is metabolized by the former. They suggested that the ability of strain Bn to prevent accumulation of the oxide could account for the observed difference between strains in their tolerance to nitrate and nitrite. Atkinson (123) had implicated, although not with complete certainty, the depression of hydrogenase activity in the presence of nitrate to the formation of small amounts of nitric oxide. It was much more inhibitory to the hydrogenase activity of *Hydrogenomonas facilis* than any of the other possible nitrate reduction products tested. Nitric oxide is formed by some denitrifying organisms and is also an inhibitor of *Proteus* hydrogenase [Krasna & Rittenberg (124)]. The claim by Keilin (125) that of various substances examined, only nitric oxide and cyanide ion were capable of coordinating with some of the valency forms of iron of heme and hematin or their hemoproteins, offers a plausible mechanism for the inhibitory action of nitric oxide. McNall & Atkinson (126) showed that *E. coli* strain Bn also utilized hyponitrite, hydroxylamine, or nitrous oxide as a sole nitrogen source. The rates of adaption suggested that nitrous oxide was not an intermediate in nitrate reduction. Senez, Pichinoty & Konovaltchikoff-Mazoyer (127) and Pichinoty & Senez (128) described the reduction of nitrite and hydroxylamine to ammonia by cell suspensions or extracts of *Desulfovibrio desulfuricans* in a hydrogen atmosphere. The uptake of molecular hydrogen corresponded to stoichiometric expectations (three moles of $H_2$ per mole of nitrite, and one mole of $H_2$ per mole of hydroxylamine). Cytochrome-$c_3$, by virtue of its ability to restore the systems inhibited by hexadecyltrimethyl-ammonium bromide, was implicated as a component. Senez & Pichinoty stressed the point that reduced cytochrome-$c_3$ was rapidly oxidized by hydroxylamine in the absence of added cells or extracts.[3] Krasna & Rittenberg (129) also observed the reduction of nitrite and hydroxylamine to ammonia by the same species with a hydrogen gas uptake consistent with reduction to ammonia. Wickerham (130) isolated yeasts with sporulation characteristics of the genus *Debaryomyces* capable of assimilating nitrite but not nitrate. He stated that although the ability to assimilate nitrate is an important test used in classifying yeasts, there

[3] Senez, J. C., and Pichinoty, F., personal communication. Subsequently published: *Biochim. et Biophys. Acta*, **28**, 355 (1958).

are no previous reports of yeast which assimilate nitrite but not nitrate. Virtanen & Saris (131) reported the oximes of pyruvic, ketoglutaric, oxalacetic, and glycolic acids to constitute 70 per cent of the "bound hydroxylamine" upon reduction of nitrite by *Torulopsis utilis* yeast. "Bound hydroxylamine" was proposed as the end product of a physiological mechanism to trap hydroxylamine in those cases where it was not reduced to ammonia quickly enough. Presumably, oximes rapidly decreased in the cells, caused either by their reduction to amino acids, or by their hydrolysis to hydroxylamine and subsequent reduction to ammonia. In this connection it is worth noting that Seaman (131a) partially purified an enzyme from the ciliated protozoan, *Tetrahymena pyriformis*, capable of catalyzing the hydrolysis of pyruvic oxime to hydroxylamine and pyruvic acid. The enzyme is inhibited by $Zn^{++}$, $Co^{++}$, $Fe^{++}$, and azide, and also hydrolyzes α-ketoglutaric oxime. Fedorov & Ilina (132) reported the ready reduction of nitrate and nitrite by soil actinomycetes to ammonia derivatives with hydroxylamine as a probable intermediate. Kessler (133, 134, 135) studied the reduction of nitrite by the green alga *Ankistrodesmus braunii* in the dark after adaptation to molecular hydrogen. The rapid reduction of nitrite, with three moles of $H_2$ being consumed for every mole of nitrite reduced, was in sharp contrast to the slow reduction of nitrate. *Scenedesmus obliquus* "D,"-containing hydrogenase, behaved similarly while *Chlorella pyrenoidosa*, lacking hydrogenase activity, was unable to use $H_2$ for the reduction of nitrite or nitrate. Nitrite reduction was inhibited by 2, 4-dinitrophenol in *A. braunii* but the reduction of nitrate to nitrite was not affected. In the light, the rate of reduction of nitrite was doubled by the presence of carbon dioxide. Vanecko & Varner (136) identified oxygen as the gas evolved upon exposure to light of nitrite-infiltrated wheat leaves. They concluded that photolysis of water was the primary and immediate source of reducing substance in the light reduction of nitrite since one and one-half moles of oxygen were evolved per mole of nitrite reduced:

$$2HNO_2 + 2H_2O \rightarrow 2NH_3 + 3O_2$$

The rate of nitrite reduction in the dark was less than 5 per cent of that in the light. Nitrite was reduced to the amino level and probably incorporated into proteins since there was no significant increase in the levels of ammonia and amide. Kessler (135) also concluded that nitrite reduction was more directly affected by the photochemical processes in *A. braunii*, whereas the effect of light upon nitrate reduction was indirect, probably in the formation of carbon compounds.

The report by Medina & Nicholas (137) of interference by reduced pyridine nucleotides in the diazotization of nitrite was preceded by similar studies and observations by Evans & McAuliffe (138), Chung (139), and Nason (140). The phenomenon was investigated by one of us (A. N.) about six years ago (unpublished results) and the study resulted in the use of a number of precautions to minimize this effect, e.g., use of catalytic quantities of pyridine nucleotide with an enzymatic-reducing system, enzymatic

oxidation of the reduced pyridine nucleotides before diazotization, etc. Garner *et al.* (141) devised a microbiological method for estimation of nitrate based on the formation of nitrite by a bacterium isolated from sheep rumen. Various methods for the determination of nitrate and nitrite were recently reviewed in detail by Nicholas & Nason (142).

*Hyponitrite and hydroxylamine reduction.*—Medina & Nicholas (121, 143, 144) reported the presence of a DPN-hyponitrite reductase in crude extracts of *Neurospora crassa*. The enzyme, designated as a metalloprotein with a flavin requirement, catalyzed the conversion of hyponitrite to ammonia and was inhibited by naphthoquinone and 2-*n*-heptyl-4-hydroxyquinoline, N-oxide, as was nitrite reductase, suggesting the participation of a heme-type compound. The authors prematurely concluded that since dinitrophenol inhibited this system (and nitrite reductase) "a phosphorylation was involved in this reducing step." Hyponitrite reductase had the same characteristics as nitrite reductase and the claim by Medina & Nicholas (144) that the enzymes are different, based on a small decrease of the latter under zinc-deficient conditions, must await further study. The fact that both enzymes were decreased in copper- or iron-deficient *Neurospora* was construed as evidence that these metals were components of the enzymes. Zinc, however, was not considered to be a component of the nitrite enzyme, although the activity decrease (30 per cent) was regarded as significant in zinc-deficient mycelia to serve as a basis for claiming both reductases to be separate enzymes. At most the data are suggestive, as is their report that manganese is a component of *Neurospora* hydroxylamine reductase based only on a deficiency effect. Manganese was already shown to be a highly specific activator of nitrite reductase and hydroxylamine reductase from soybean leaves [Nason, Abraham & Averbach (120)] and of hydroxylamine reductase from *Azotobacter* [Spencer, Takahashi & Nason (85)]. Bulen (145) also reported a particulate pyridine nucleotide-hydroxylamine reductase in *Azotobacter vinelandii*. That hyponitrite is an intermediate in nitrate reduction was suggested by Vanecko & Frear (146) in view of the conversion of $N^{15}$-labeled hyponitrite into reduced nitrogen fractions by green leaves exposed to light.

Reinvestigation of "ammonium dehydrogenase" in *Bacillus pumilis* extracts which were reported to catalyze the reversible reduction of hydroxylamine by DPNH to ammonia, DPN, and water [Klausmeier & Bard (147)], showed that the system had only hydroxylamine reductase activity similar to that of *Neurospora* [Zucker & Nason (119)]. Roussos, Takahashi & Nason (148), while confirming the observations of Klausmeier & Bard, found that the apparent enzymatic reduction of DNPH by $NH_4OH$ resulted instead from an indirect pH effect. The results were in keeping with the calculated equilibrium constant, $10^{35}$, for hydroxylamine reduction by DPNH, making it unlikely that the reverse reaction could be demonstrated. Kono, Taniguchi & Egami (149) purified a soluble, autoxidizable and carbon monoxide-binding cytochrome from a halotolerant bacterium. The

pigment catalyzed the reduction of hydroxylamine by reduced methylene blue which was stimulated by $Mn^{++}$. Ishimoto, Yagi & Shiraki (150), using cell-free extracts of sulfate-reducing bacteria, showed a cytochrome requirement for the reduction of hydroxylamine to ammonia by hydrogen. Chemically reduced cytochrome also evolved hydrogen gas in the presence of hydrogenase.

*Reduction of organo-nitro compounds and derivatives.*—The metabolic significance of the reduction of organo-nitro compounds by intact cells and purified enzyme preparations is still obscure. The possibility that nitrate assimilation may proceed by way of an organic pathway whereby an inorganic reduction product of nitrate other than ammonia is introduced into organic compounds and further reduced, was postulated by McElroy & Spencer (151). The experiments of Silver & McElroy (152), using *Neurospora* mutants blocked at different genetically controlled steps in nitrite utilization, suggested a pathway leading from hydroxylamine through pyridoxal oxime phosphate and pyridoxamine phosphate to amino nitrogen. The mutants required pyridoxine in the media for growth on nitrite but not for growth on ammonia. They proposed that an alternative inorganic assimilative pathway via hydroxylamine and ammonia supplemented the organic route. McElroy & Spencer (151) also considered the possibility that the pyridine nucleotide-nitroaryl-reducing enzyme might be utilized in the organic pathway. The characteristics of a number of enzymes including xanthine oxidase, diaphorase, and pyridine nucleotide-linked flavoproteins in catalyzing the reduction of various nitroaryl compounds (including nitrofurazone and chloramphenicol) with varying degrees of specificity were discussed in the review by Nason (80). The possibility was presented that nitroaryl compounds, like methylene blue and indophenol, are simply nonspecific electron acceptors for flavoprotein systems. The work of Saz, Brownell & Slie (153) and Saz & Martinez (154) with a nitro-reductase from chlortetracycline-resistant and -sensitive *E. coli* strains is of interest. The enzyme is a $Mn^{++}$-activated, pyridine nucleotide-flavoprotein catalyzing the reduction of nitro groups of chloramphenicol and *p*-nitrobenzoic acid. The nitro-reductase isolated from chlortetracycline-sensitive *E. coli* was inhibited by low concentrations of chlortetracycline whereas the enzyme from resistant cells was unaffected. The easily dissociable FMN of the chlortetracycline-sensitive enzyme, as well as the striking stimulation by $Mn^{++}$ in contrast to the tightly-bound FMN and small activation effect by $Mn^{++}$ of the resistant enzyme, suggested a mechanism of action for the antibiotic. They postulated that chlortetracycline, as a potent chelating agent, was binding the easily dissociable $Mn^{++}$ of the sensitive enzyme. In development of resistance, an altered protein was presumably synthesized with a stronger binding capacity than chlortetracycline for $Mn^{++}$ and possibly flavin. Saz & Martinez (155) observed a similar relationship for the $Mn^{++}$-activated, flavin-containing nitro-reductase from chlorotetracycline-sensitive and -resistant *E. coli* cells. Asnis (156) reported that cell-free extracts of nitro-

furazone-resistant *E. coli* carried on a single type of nitrofurazone reduction characterized by the disappearance of a typical absorption peak at 375 mμ with the appearance of a new peak at 355 mμ, whereas extracts of nitrofurazone-susceptible cells had an additional reduction mechanism. The latter, which was also mediated by a pyridine nucleotide-linked FMN enzyme, involved the disappearance of an absorption peak at 375 mμ without the appearance of any new absorption peak in either the visible or ultraviolet range. This type of reductive activity was suggested to play a role in the antibacterial action of nitrofurazone, perhaps by leading to free radical production. Tsukamura (157) found that the reduction of picric acid to picramic acid by resting cells and cell-free extracts of *Mycobacterium avium* was accomplished as well by an L-amino acid oxidase preparation from these cells. A DPN-cytochrome-*c* reductase from *M. avium* and from rabbit liver also reduced picric acid. The color changes which nitroprusside undergoes in the presence of the *Proteus vulgaris* hydrogenase system was attributed to its reduction from $(Fe(CN)_5(NO))^{=}$ to $(Fe(CN)_5(NOH))^{\equiv}$ [Krasna & Rittenberg (158)]. The hydrogenase of *Desulfovibrio desulfuricans* but not of *Rhodospirillum rubrum* also reduced the compound. The reduction of methemoglobin by extracts of *Achromobacter fischeri* and *E. coli* arose from a diaphorase or flavoprotein with an easily dissociable flavin, according to Cormier & Rostorfer (159). The partially purified enzyme from *E. coli* resembled the methemoglobin reductase from red blood cells, the $FMNH_2$ nonenzymatically transferring its electrons to methemoglobin. The inhibition by methemoglobin of the cell-free luminescent system of *A. fischeri* was attributed to a competition with luciferase for the hydrogens of $FMNH_2$. The findings of Kiese, Resag & Schneider (160) which indicate that purified methemoglobin reductase from red blood cells and old yellow enzyme reduced nitrosobenzene, and the fact the latter enzyme also reduced methemoglobin, support the proposed action of organo-nitrogen compounds (and methemoglobin) as nonspecific electron acceptors in many flavoprotein systems. Westerfield, Richert & Higgins (161) concluded that most, if not all, enzymes in rat tissues capable of reducing the nitro group of *p*-nitrobenzene sulfonamide were molybdenum-dependent enzymes. A number of such different enzymes were present with any one system exhibiting some specificity towards different nitro substrates. They used the technique of administering tungstate as a means of removing molybdenum and molybdenum enzymes from tissues. Jensen & Gundersen (162) isolated an atypical form of *Corynebacterium simplex* which decomposed 4,6-dinitro-*o*-cresol and several related compounds with the release of nitrite. An enzyme system from pea plants which catalyzed the oxidation of 2-nitro-propane by oxygen with the release of nitrite and acetone has been described by Little (163).

## Nitrification

The process is generally defined as the oxidation of ammonium ion and other forms of nitrogen to nitrite and nitrate by autotrophic organisms. A

strict interpretation would restrict nitrification only to autotrophs, as typified by *Nitrosomonas* and *Nitrobacter,* since they obtain energy by the oxidation of ammonium and nitrite ions. However, as already indicated by Delwiche (164) in a review of nitrification, a number of heterotrophic microorgansms oxidize ammonia and other reduced forms of nitrogen to nitrite and nitrate, frequently on a smaller scale than the autotrophs. A broader view of the definition would include these heterotrophs, thus suggesting two general groups, the Autotrophic Nitrifyers and Heterotrophic Nitrifyers, an interpretation which the present reviewers favor. Fisher, Fisher & Appleman (165) isolated a number of Gram-negative, short-rod soil bacteria which produced nitrite from ammonia. The small amount of nitrite formed by these heterotrophs as compared to that by autotrophic organisms suggested "that heterotrophic nitrification is not an important energy-yielding mechanism for these bacteria." However, in view of the prevalence of these morphological types in the soil, they believed that these bacteria contributed substantially to the nitrogen cycle in nature. Ruban (166) indicated that the inhibition by organic compounds of nitrification and growth of *Nitrosomonas* is temporary since the cells were viable and resumed activity when transferred to an inorganic medium. Alleem, Engel & Alexander (167) found ammonia at alkaline pH to be a potent inhibitor of nitrite oxidation by *Nitrobacter,* but not of nitrification of ammonia by *Nitrosomonas.* Klein (168) found that fully developed nitrifying bacteria could be stored at room temperature for three to seven months when covered with paraffin oil, whereas Gundersen (169) had limited success using a culture immediately after inoculation under similar conditions. Molybdenum and flavin were implicated in nitrification by *Nitrobacter* by Zavarzin (170) who observed a stimulation by sodium molybdate especially in the presence of riboflavin. Our meager knowledge of the biochemical pathway and mechanisms of autotrophic nitrification, resulting, in large part, from our inability to grow adequate quantities of cells, has not advanced substantially in the last three years. With regard to elucidation of intermediates there has been no progress beyond the evidence for hydroxylamine as an intermediate. The latter was based in large part on the work of Hoffman & Lees (171) who demonstrated that hydrazine inhibited oxidation of ammonium ion by *Nitrosomonas,* resulting in the accumulation of hydroxylamine. Hydroxylamine at low concentrations was already shown to undergo oxidation by these cells as rapidly as was ammonium ion. The oxidation of pyruvic oxime and other oximes had also been demonstrated by a number of workers [see Delwiche (164)]. Lees & Simpson (172), continuing their work on the inhibition by chlorate of nitrite oxidation by *Nitrobacter,* concluded that chlorate did not inhibit nitrate oxidation directly but was converted into an inhibitory compound, possibly chlorite. Inhibition was not reversed by washing the cells with water whereas cyanate inhibition was. The proportionality of nitrite oxidation to the cytochrome (551 mμ) content of the cells suggested that cytochrome was initially concerned with nitrite oxidation by

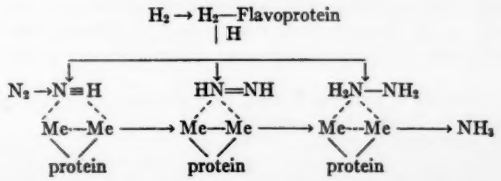
*Nitrobacter.* Of particular interest are the reports of cell-free oxidation of ammonia and hydroxylamine to nitrite by autolysates and filtrates of *Nitrosomonas europea* [Imshenetskii, Ruban & Buzina (173); Imshenetskii, Ruban & Artemova (174); Imshenetskii & Ruban (175)]. The crude enzyme retained its power even after "brief boiling" but was inactivated by boiling for 30 min. The report of a strictly autotrophic bacterium, belonging to the Actinomycetaceae, capable of growing on KCN as a source of nitrogen and carbon with the production of ammonia is included in the present section for lack of a more appropriate place [Ware & Painter (176)]. All major areas in the biochemistry of nitrifying organisms remain to be clarified, including the intermediates and mechanisms of the nitrifying pathway, other principal metabolic pathways, the mechanism of energy coupling in the "autotrophic nitrifyers," and the reason for their inability to grow heterotrophically.

## Nitrogen Fixation

The pathway and mechanisms for the assimilation of molecular nitrogen still remains one of the most formidable problems in biology. In the field of inorganic nitrogen metabolism the process of nitrification may well prove to be of equal difficulty, although it has received only a small fraction of the research effort devoted to nitrogen fixation. Since the presentation of the comprehensive reviews on various aspects of nitrogen fixation at the McCollum-Pratt Symposium by Gest, Judis & Peck (177), Burris (178), and Shug, Hamilton & Wilson (179), there has been the characteristically slow painful progress in the area.

*Cell-free nitrogen fixation.*—The two reports by Wilson's group at the University of Wisconsin of nitrogen fixation with sonic extracts of *Clostridum,* root nodules, and *Azotobacter* are the only positive claims in an otherwise unmarred picture of failure by others to obtain nitrogen fixation consistently at the cell-free level. The earlier study by Shug, Hamilton & Wilson (179), using *Clostridum pasteurianum* extracts, showed a two-banded difference spectrum with maxima at 450 and 390 mμ upon exposure to 0.2 atm. of hydrogen gas followed by admission of molecular nitrogen to atmospheric pressure. This was interpreted as a reduction of endogenous flavin by molecular hydrogen with subsequent oxidation by nitrogen gas. The peaks in the Soret region at 405 and 425 mμ were presumed to be reduced nitrogen products. The more recent report by Hamilton, Shug & Wilson (180) pointed similarly to a reduction of flavins, and cytochromes-$a_1$, $a_2$, *b,* and *c* by hydrogen gas in the presence of an *Azotobacter* extract with a subsequent specific oxidation of the flavins and cytochrome-*b* by molecular nitrogen. Spectral examination of soybean nodule extracts indicated an electron carrier role for hemoglobin since nitrogen gas oxidized endogenous hemoglobin to hemiglobin, whereas the opposite reaction was observed with molecular hydrogen. A possible experimental pitfall would be an oxygen contamination in the nitrogen gas. This apparently was ruled out by prior chromous chloride removal of oxygen. The results found by Wilson and his co-workers experimentally implicate for the first time a mechanism for nitro-

gen fixation. Characterization of these systems will be awaited with interest. The experiments with the nodule extracts reflected the action of a hydrogenase although the slight deuterium exchange reaction appeared negligible. The data of Hoch, Little & Burris (181) had already suggested the presence of hydrogenase activity in soybean root nodules. The above evidence also strengthens the possibility of a relation between hydrogenase and nitrogen fixation. It was previously established that the hydrogenase content of *Azotobacter* cells was markedly greater in cells which were fixing nitrogen [Lee & Wilson (182)], although in certain anaerobic nitrogen fixers, for example, *C. pasteurianum,* no such obvious relation existed [Gest, Judis & Peck (177)]. *Rhodospirillum rubrum,* the first of the photosynthetic bacteria found to be capable of nitrogen fixation, is an anaerobic nitrogen fixer whose hydrogenase activity is higher when grown on molecular nitrogen than on glutamate or ammonia [Gest, Judis & Peck (177)]. Molecular hydrogen, already established as a competitive inhibitor of nitrogen fixation in *Azotobacter,* other aerobic agents, and in red clover nodules at high concentrations (0.8 atm.), inhibited the presumed nitrogenase extract from *C. pasteurianum* [Shug, Hamilton & Wilson (179)]. Recently, Hiai *et al.* (183) reported the competitive inhibition by hydrogen gas of anaerobic nitrogen fixation in *Clostridium,* with a 60 per cent decrease in nitrogen fixation at 0.8 atm. $H_2$ and 0.2 atm. $N_2$. The apparent dissociation constant of the $H_2$-enzyme complex was approximated to be 0.1 atm., a value similar to that obtained by others for aerobic nitrogen fixation. The results are contrary to the older data of Rosenblum & Wilson (184) who indicated that the rate of anaerobic nitrogen fixation was not appreciably influenced by the presence of molecular hydrogen. The present findings of Hiai *et al.* serve to unify the general action of molecular hydrogen as a competitive inhibitor of both aerobic and anaerobic nitrogen fixation. The hypothesis that hydrogen gas is an electron donor in the fixation process and, at higher tensions, competes with molecular nitrogen for the active sites of nitrogenase, was advanced by a number of workers. Possible mechanisms for the interaction of hydrogenase and the hypothetical nitrogen-fixing enzyme (nitrogenase) were proposed by Winfield (185) and by Shug, Hamilton & Wilson (179), involving chemisorption of molecular nitrogen to a metallo-enzyme containing 2 metal atoms, possibly molybdenum or iron which form single or double covalent bonds to nitrogen. Another enzyme, presumably a flavoprotein such as hydrogenase would transfer hydrogen atoms to the chemisorbed nitrogen with the ultimate release of the nitrogen as ammonia.



The report by Nason *et al.* (186) of nitrogen fixation by washed particles of sonicated *Azotobacter* cells was attributed in part to a small percentage of intact cells. Dilution of *Azotobacter* cells resulted in a striking increase in nitrogen fixation per cell (unpublished results).[4] Jose & Pengra (187), using *Azotobacter* protoplasts prepared by treatment with lysozyme and versene, observed a small incorporation of $N^{15}$ gas (0.04 atom per cent excess as compared with 0.83 per cent excess for whole cells). All attempts to attain nitrogen fixation at the cell-free level point to the extreme lability of the system, a situation reminiscent of oxidative phosphorylation. This possibly reflects the delicately integrated physical organization of the nitrogen fixing system.

*Intermediates.*—Allison & Burris (188), in a kinetic analysis of $N^{15}$ accumulated in the cells and medium of *Azotobacter* cultures, concluded that the sequence of nitrogen fixation was by way of ammonia to glutamic acid. They indicated that the shapes of the ammonia and dicarboxylic acid curves suggested a typical precursor-product relationship with ammonia appearing as a product of nitrogen fixation before the dicarboxylic amino acids. However, between the two-minute and five-minute steady-state exposures to $N_2{}^{15}$, glutamic acid attained a higher $N^{15}$ concentration than free ammonia, which appeared incompatible with the postulated sequence of nitrogen fixation. They attributed this to a "possible randomization of compounds between intercellular and extracellular pools when the organisms were inactivated and harvested." No oxidized nitrogenous compounds were isolated and it was postulated that the $N_2$ fixation by *Azotobacter* was entirely a reductive process, occurring at active sites on, or very close to, the cell surface. Burma & Burris (189), studying the kinetics of ammonia utilization by *Azotobacter,* observed that cells actively fixing nitrogen used ammonia immediately. After a one-minute exposure to $N^{15}H_4{}^+$ all isolated amino acids, purines, and pyrimidines contained $N^{15}$. They suggested that cells which utilize molecular nitrogen, form $NH_4{}^+$ as an intermediate. Another explanation is that the enzymes for ammonia utilization are constitutive and may have no direct relation to nitrogen fixation. They found that the distribution of $N^{15}$ from $N^{15}H_4{}^+$ was similar to that observed earlier when $N_2$ was supplied to *Azotobacter.* Mozen & Burris (190) ruled out nitramide as an intermediate in nitrogen fixation, assuming the initial step to be an oxidation, and as a source of nitrogen for *Azotobacter.* Aside from the rapid decomposition of nitramide in solution to water and nitrous oxide they observed no incorporation of $N^{15}$ in cells exposed to labeled nitramide. More recent evidence also tends to exclude hydroxylamine as an intermediate in nitrogen fixation, although not conclusively. The data of Spencer, Takahashi & Nason (85), showing that formation of the nitrite and hydroxylamine reductases of *Azotobacter* was stimulated by nitrate but not by

[4] Similar increases in nitrogen fixation have also been observed by R. H. Burris and his co-workers (personal communication).

molecular nitrogen, ammonium sulfate, or glutamate argued against nitrite and hydroxylamine as intermediates in nitrogen fixation. Virtanen and his co-workers at one time regarded hydroxylamine as an intermediate on the direct pathway of nitrogen fixation in legumes followed by condensation with α-ketocarboxylic acids and reduction to amino acids [see review by Gest, Judis & Peck (177)]. The Wisconsin group showed, however, that *Azotobacter* failed to use exogenous hydroxylamine and utilized oximes only poorly [Burris 178]. Recently, Virtanen's group also provided evidence against their hydroxylamine theory (190a, 190b).

The above postulated relation between hydrogenase and nitrogen fixation implicates hydrazine as an intermediate in nitrogen fixation. Bach & Burris (191) and Bach (192) supplied hydrazine to dense *Azotobacter* suspensions and found that most of the hydrazine which disappeared was recovered as the cyclized product of the chemical condensation of hydrazine with α-ketoglutarate, identified as 3,4-dihydroxypyridazinone-5-carboxylic acid (abbreviated as PCA). Extracts of *Azotobacter* and soybean nodules contained three compounds having $R_F$ values identical with PCA and with labelled compounds isolated from cells incubated with $N^{15}H_2N^{15}H_2$. Excised soybean nodules exposed to $N_2{}^{15}$ contained $N^{15}$ concentrations as high as two-atom per cent excess in the isolated PCA-type compounds. The concentration of $N^{15}$ in the PCA was frequently equal to or higher than that of glutamic acid or ammonia. Only a small fraction of $N^{15}$ was incorporated from $N^{15}H_4{}^+$ into PCA. A scheme was proposed whereby hydrazine as an intermediate of nitrogen fixation is released from the enzyme sites of nitrogenase to form PCA by condensation with ketoglutarate. The reduction of PCA would presumably yield glutamine if tetrahydro-pyridazinone-5-carboxylic acid rearranged to form *N*-amino pyrolidone carboxylic acid. The postulated scheme, however, would seem to repudiate the argument for ammonia rather than organic nitrogen as a direct product of nitrogen fixation. Azim & Roberts (193) showed that hydrazine at concentrations below $2 \times 10^{-5}$ *M* stimulated nitrogen fixation approximately 30 per cent, whereas higher concentrations gave an almost complete inhibition.

Burris, Magee & Bach (194) determined the Km for the symbiotic nitrogen-fixing system in soybean nodule slices to be 0.025 atm. $N_2$, a value very similar to that for *Azotobacter vinelandii* and the blue-green alga, *Nostoc muscorum*.

*Inhibitors.*—Rakestraw & Roberts (195, 196) observed that azide and cyanate inhibited respiration and inorganic phosphate uptake of *Azotobacter* at concentrations greater than $10^{-5}M$, while at lower concentrations they stimulated respiration. At lower concentrations only azide stimulated phosphorylation. They concluded that although azide and cyanate are structurally and electronically similar, their effects are different on bacteria. Like nitrous oxide, azide also seems to be a reversible inhibitor of nitrogen fixation, and possibly acts by competing with nitrogen gas for the active sites on nitrogenase. The observation that $10^{-4}M$ azide completely inhibited phosphate

uptake while $N_2$ fixation still continued was interpreted to mean that the mechanism of $N_2$ fixation did not involve oxidative phosphorylation in the initial stage. According to Azim & Roberts (197), nitrite at higher concentrations (exceeding $10^{-4}M$) inhibited both respiration and nitrogen fixation in *Azotobacter,* whereas at lower concentrations a striking stimulation of fixation occurred. Nitrous oxide, a specific and competitive inhibitor of nitrogen fixation, is utilized by *Azotobacter* [see Burris (178)]. Nitrous oxide and carbon monoxide inhibit anaerobic nitrogen fixation by *Clostridium* [Hino (198)]. Ammonia assimilation in this bacterium was inhibited only by carbon monoxide and to a lesser extent than nitrogen fixation. Although ammonium salts and urea strongly inhibited nitrogen fixation in *Azotomonos fluorescens,* Fedorov & Kalininskaya (199) reported that nitrate which is also used by the organism did not cause a cessation of the fixation process. In alkaline medium, from which nitrates are more easily assimilated, there was a 100 per cent inhibition of the fixation process whereas at pH 6.2 there was no effect. Bullen & Frear (200) have preliminary evidence that potassium orthophosphite is a specific inhibitor of nitrogen fixation. The compound was relatively nontoxic to intact yeast and animal cells, whereas at $0.01M$ concentration it completely inhibited nitrogen fixation in *Azotobacter.* When ammonium acetate was present, there was no inhibition of growth by $0.01M$ orthophosphite.

*Molybdenum and other metals.*—Although molybdenum is essential for organisms which are fixing molecular nitrogen, with the requirement being considerably greater than for those utilizing nitrate as a nitrogen source, the role of molybdenum in fixation is still unknown. Bové, Bové & Arnon (201) compared several species of *Azotobacter* in terms of their response to molybdenum in growth and nitrogen fixation. *A. chroococcum* failed to fix nitrogen in the absence of added molybdenum to purified medium, whereas *A. agile* and *A. vinelandii* made considerable growth and fixed nitrogen at about 50 per cent of optimum without the inclusion of molybdenum. Vanadium was reported to be capable of partly replacing molybdenum. Magee & Burris (202) purified a molybdenum-protein fraction 150-fold from *Azotobacter* grown on $Mo^{99}$. The fraction was stable to dialysis against phosphate buffer but $Mo^{99}$ could be rapidly dissociated by dialysis against cyanide solution. Addition of the molybdenum-protein to cell-free preparations did not stimulate nitrogen fixation. Keeler, Bulen & Varner (203) demonstrated that a large portion of the $Mo^{99}$ taken up by *Azotobacter* is associated with a particulate fraction but is stable against cyanide dialysis. Keeler (204) further reported that $Mo^{99}$ uptake by growing *Azotobacter* cells was influenced by aeration and the iron level of the medium, and was unaffected by the nitrogen source except for molecular nitrogen. That tungstate is a competitive inhibitor of molybdate in nitrogen fixation by *Azotobacter* was shown by Takahaski & Nason (113) and Keeler & Varner (205). Vanadium, which has been reported by a number of workers to serve in place of molybdenum in nitrogen fixation, failed to show a competitive an-

tagonism with tungstate, thus adding further support to the contention that vanadium cannot replace the molybdenum requirement. Keeler & Varner (205) found that 100 p.p.m. tungsten did not seriously inhibit growth, whereas it almost completely prevented $Mo^{99}$ uptake by *Azotobacter* grown on $N_2$ nitrate. The relatively large quantities of molybdenum taken up and combined with protein by growing cells were apparently not essential for growth. In another report Keeler & Varner (206) indicated that tungsten was incorporated into the same protein fraction as molybdenum. According to Esposito & Wilson (207) molybdenum and iron are both specifically needed for nitrogen fixation by *Azotobacter vinelandii* O, the requirement being either eliminated or spared when ammonium ion was supplied. Vanadium did not replace molybdenum in this strain. Allen (208), in a review article on photosynthetic nitrogen fixation by blue-green algae, pointed out that the molybdenum requirement of the blue-green alga, *Anabaena cylindrica,* is specific for nitrate reduction and nitrogen fixation. It cannot be replaced by vanadium. A pasture response to applications of sodium tungstate on a New Zealand soil led Davies & Stockdill (209) to suggest that tungsten could assume the role of molybdenum, presumably in symbiotic nitrogen fixation. Vanadium had no effect either by itself or in conjunction with molybdenum or tungsten.

Esposito & Wilson (207) pointed out that past work by others indicates that uncertainty exists about a calcium requirement for nitrogen fixation. They proceeded to demonstrate a specific calcium requirement for nitrogen fixation by *Azotobacter vinelandii* O, the presence of ammonia eliminating the need for added calcium. Strontium but not magnesium replaced the need for calcium. According to Allen & Arnon (210), calcium is essential for growth of the blue-green alga, *Anabaena cylindrica,* on nitrate or nitrogen gas and cannot be replaced by strontium. Esposito & Wilson (211) believe that calcium is in an interrelationship with phosphate during nitrogen fixation in *A. vinelandii,* making it essential for the synthesis of polymetaphosphate. They suggested that metaphosphate is the required energy source for nitrogen fixation. Esposito (212) observed that the addition of calcium during the lag phase of calcium-deficient *A. vinelandii* stimulated the rate of phosphate exchange between exogenous $P^{32}$-phosphate and the trichloroacetic acid-soluble mononucleotide fraction and fractions associated with the acid insoluble metaphosphate of the cell. When ammonium acetate was added to the cultures, there was no significant difference in the rate of phosphate exchange. Wyss & Chu (213) reported that the inhibition in growth of *Azotobacter agile* by high concentrations of either strontium or magnesium salts was relieved by calcium at molar ratios of about 1 to 100 for strontium and 1 to 10,000 for magnesium. Keeler & Varner (214) found no correlation between $Si^{31}$ and $Mo^{99}$ in uptake and distribution studies with *A. vinelandii.* They concluded that silicate was not involved in molybdate metabolism of these cells as a silicomolybdate complex. Arnon & Allen (215) regarded sodium as essential for growth of the blue-green alga, *Anabaena*

*cylindrica*. In most experiments nitrogen gas served as the nitrogen source and neither potassium, lithium, rubidium, nor cesium substituted for sodium.

*Metabolism of nitrogen-fixing organisms.*—Tissières & Burris (216) and Tissières (217) purified and characterized two cytochromes from *A. vinelandii* which resembled mammalian cytochrome-*c* in several important properties and which they called cytochromes-$c_4$ and -$c_5$. These cytochromes were found to react only with the oxidase from *A. vinelandii*. Wilson & Wilson (218) had earlier prepared from the same organism a particulate succinoxidase system which oxidized DPNH and contained cytochrome different from mammalian cytochrome-*c*. Alexander & Wilson (219) reported that a cytochrome-rich particulate preparation from *A. vinelandii* had cytochrome oxidase, DPNH oxidase, succinic dehydrogenase, and malic oxidase activities exclusively. The supernatant solution contained aconitase, isocitric dehydrogenase, and α-ketoglutarate oxidase. Breummer *et al.* (220) purified a particulate system from the same organism which they designated as the electron transfer particle. It catalyzed the oxidation of succinate and DPNH and contained flavin, heme, nonheme iron, and copper. Layne & Nason (221) obtained from *A. vinelandii* a soluble cytochrome oxidase capable of oxidizing reduced mammalian cytochrome-*c*, although at a slower rate than reduced cytochromes-$c_4$ and -$c_5$ of *Azotobacter*. The ability of this system to oxidize reduced mammalian cytochrome-*c* was demonstrated only after removal or inactivation of a naturally occurring inhibitor in the *Azotobacter* preparation. It was not determined whether the oxidation of the mammalian cytochrome proceeded directly or indirectly by way of the endogenous *Azotobacter* cytochrome. Thorogood (222) isolated three and possibly four ferro-protoporphyrin proteins from soybean nodules. The absorption spectra of the oxygenated ferro-pigments were typical of oxygenated hemoglobins. There are a number of reports of coupled oxidative phosphorylation in cell-free *Azotobacter* preparations [Rose & Ochoa (223); Tissières, Hovenkamp, & Slater (224); Josten (225); Hartman, Brodie & Gray (226)]. The latter workers demonstrated that this extremely labile system was stabilized by the addition of ATP and $Mg^{++}$.

Bonnier & Sironval (227) concluded that day length to which leaves of plants were exposed had an influence on nodule formation and their nitrogen-fixing capacity. A 16-hr. day resulted in heavy and numerous effective nodules with a dark red color in their internal tissue, in contrast to the poor yields with an 8-hr. day. Monsour & Wyss (228) isolated three mutants of *Azotobacter* which required leucine for growth and nitrogen fixation.

*Ubiquity of nitrogen fixation.*—During the past few years an increasing number of organisms have been shown to fix gaseous nitrogen. In addition to *Azotobacter* and *Clostridium* species, legume nodules, photosynthetic bacteria such as *Rhodospirillum rubrum, Chlorobium* species, *Chromatium* species, the blue-green algae *Nostoc muscorum* and *Anabaena cylindrica,* nitrogen fixation occurs in a number of other organisms. These include *Aerobacter aerogenes, Methanobacterium omelianskii,* and soil yeasts such

as *Saccharomyces* and *Rhodotorulla* species [see Brown & Metcalfe (229) for references]. These authors observed that a black, yeast-like organism, isolated from soil and identified as a species of *Pullularia*, fixed molecular nitrogen. Hamilton & Wilson (230), maintaining the pH at physiological values, markedly increased nitrogen fixation in *Aerobacter aerogenes*. The addition of a small amount of fixed nitrogen was necessary to initiate fixation. Jensen (231) also reported higher values for nitrogen fixation by two strains of *A. aerogenes*. A greater amount of nitrogen was fixed at lower temperatures (15° to 18°C.) than at higher temperatures (25° to 30°C.) in proportion to the nutrients consumed although the growth rate was higher at higher temperatures. A growth factor in yeast or agar was necessary for fixation. Anderson (232) isolated a soil bacterium designated as *Pseudomonas azotocolligans*, which fixed nitrogen. Rakhno & Tokhuer (233) obtained a nitrogen-fixing, thermophilic bacterial culture from Esthonian soil with optimal growth rates at 45° to 50°C. A Gram-negative aerobic bacterium capable of growth and nitrogen fixation in acid media (pH 4.0) was isolated from soils cropped with jute [Roy & Mukherjee (234)]. *Azotobacter indicum*, which was segregated from other *Azotobacter* and placed in the new genus *Beijerinckia*, has been discussed by Kluyver & Becking (235) and by Ruinen (236). It grows and fixes nitrogen within the remarkably wide pH range of 3.5 to 9.0 and is restricted to the tropics. Allen (208) indicated that nitrogen fixation by the blue-green alga *Anabeana cylindrica* differs from that of heterotrophic organisms such as *Azotobacter* in that it is not prevented by combined nitrogen in the medium. She pointed out that no marine nitrogen-fixing, blue-green algae have as yet been isolated. An earlier paper by Watanabe, Nishigaki & Konishi (237) reported that of 643 samples collected from various far eastern and south sea regions, only 13 blue-green algae species fixed nitrogen. They belonged to the genera *Tolypothrix*, *Nostoc*, *Schizothrix*, *Calothrix*, *Anabaenopsis*, and *Plectonema*. *Tolypothrix tenuis*, *Calothrix brevissima*, *Anabaenopsis* species, and *Nostoc* species were the only nitrogen-fixing organisms which proved to be bacteria-free. Kratz & Myers (238) were unsuccessful in growing the blue-green algae *Anabaena variablis* and *Anacystis nidulans* in nitrogen-free media. Bond & Scott (239) demonstrated nitrogen fixation in the lichens *Collema granosum* and *Leptogium lichenoides*. *Nostoc* is the algal component of both forms. Similar results were shown for the liverwort *Blasia pusilla* which contains *Nostoc* in the thallus cavities. The algae appeared to be the agent responsible for $N_2^{15}$ incorporation. There was no evidence for nitrogen fixation in the mycorrhizal roots of intact plants of *Calluna vulgaris* or of *Pinus sylvestris*, both of which were previously reported to fix nitrogen. Further evidence for the occurrence of nitrogen fixation by root nodules of alder (*Alnus glutinosa*) was provided by Bond (240), Virtanen (241), and Bond & Gardner (242). The latter workers indicated that nitrogen fixation was associated with the nodule-bearing plants of bog myrtle (*Myrica gale*) and sea buckthorn. Bond (243) observed that although detached nodules of

*Alnus glutinosa* fixed less total $N_2^{15}$ as compared with attached nodules, the amounts considerably exceeded that observed with detached legume nodules on the basis of fixation per unit of total nodule nitrogen. Bond, MacConnell & McCallum (244) inoculated *Hippophae rhamnoides* with a suspension of crushed nodules, and showed that the subsequently developed root nodules fixed nitrogen. Khudiari (245) found that *Prospopis stephaniana,* a dominant wild leguminous plant in Iraq, contained reddish root nodules. The latter possessed large amounts of nitrates "the presence of which is attributed to fixation of atmospheric nitrogen by bacteria." The bacterium may be a new species of *Rhizobium.*

Parker (246) envisaged symbiotic nitrogen fixation in plants as a stepwise evolutionary development. He speculated that the major steps involved, first, a casual association between free-living, nitrogen-fixing bacteria and plants on the surface of roots followed by a loose symbiosis on the root surfaces. Presumably, the bacteria then entered the cortical tissues of the root and continued the symbiotic relationship. Finally, a specialization of the root tissue occurred, resulting in the present-day organized nodule.

## Concluding Remarks

The essential feature of the metabolic interconversions of the various inorganic nitrogen forms and, to a certain extent, of the organo-nitro compounds and their derivatives, is the oxidation-reduction change in the state of the nitrogen atom ranging between the extreme of plus five and minus three. Thus, nitrate assimilation, nitrate respiration, and denitrification (a form of nitrate respiration) are the result of the stepwise reduction of nitrate to the more negative oxidation states of nitrogen. Nitrogen fixation is characterized by the activation of the inordinately stable bond of molecular nitrogen and subsequent reduction to the oxidation state represented by ammonia or the amino group. Nitrification in its overall effect is a reversal of nitrate assimilation, namely, the oxidation of ammonia to nitrate.

An important difference between nitrate respiration and nitrate assimilation is that the former is presumably coupled to high-energy phosphate esterification since nitrate can take the place of oxygen as a terminal electron acceptor. As yet there is no direct evidence, however, that oxidative phosphorylation does occur under these circumstances. This important difference between the two types of nitrate reduction is probably reflected in the properties of their nitrate-reducing systems. The nitrate reductase of nitrite assimilation is a molybdoflavoprotein, whereas the nitrate reductase of nitrate respiration appears to be a cytochrome system. The latter bears more structural resemblance to the established terminal oxidative phosphorylation system in animal mitrochondria and very likely in bacterial preparations. Isolation and characterization of the presumed coupled oxidative phosphorylation system from bacteria capable of nitrate respiration could provide the decisive experimental evidence. A study of cell-free preparations derived from the autotrophic nitrifyers such as *Nitrosomonas*

and *Nitrobacter* would be of great importance. While nitrification has not yet been achieved in bacterial extracts, resulting in part from the difficulty in growing large numbers of the organisms, the evidence is more conclusive that this process is coupled to the trapping of useful energy. The mechanisms of oxidation-reduction in nitrification obviously do not involve a stepwise reversal of the nitrate assimilation process. The pyridine nucleotides could not be involved as electron carriers directly in the pathway of nitrification as they are in nitrate assimilation, in view of their unfavorable potentials. If they were concerned, then the reactions would have to be endothermic rather than exothermic. The demonstration by Heppel & Porterfield (247) of the oxidation of nitrite to nitrate by the peroxidase-like action of liver catalase offers a possible model for a nitrification mechanism. In order to meet the requirements for autotrophic nitrification it would also have to be coupled to an energy-trapping system such as phosphate esterification. Coupled phosphorylation, however, has never been shown to occur concomittantly with catalase or peroxidase action.

Aside from the inherent difficulties of attaining nitrogen fixation at the cell-free level, the problem is severely limited by a slow, tedious, and expensive $N^{15}$ assay method. This has necessarily discouraged a wide exploration of possible attacks on the problem despite the high sensitivity of the determination. While the application of activation analysis does not seem promising at this time, there is some hope that the successful use of nuclear resonance with $N^{15}$ will considerably shorten the labor and time demanded by the mass spectrometer procedure.

The last six years have witnessed rapid advances in our knowledge of inorganic nitrogen metabolism as they have in most areas of biochemistry. Most of the progress has been made at the enzymatic level, and there is little question that the further clarification of the area of inorganic nitrogen metabolism will stem largely from the study of cell-free preparations. This will be the necessary preparation for a better understanding of these processes as they occur within the living cell.

## LITERATURE CITED

1. Wells, A. F., *Structural Inorganic Chemistry*, 2nd ed. (Oxford University Press, New York, 727 pp., 1950)
2. Virtanen, A. I., *Ann. Rev. Microbiol.*, **2**, 485 (1948)
3. Wilson, P. W., and Burris, R. N., *Ann. Rev. Microbiol.*, **7**, 415 (1953)
4. Lochhead, A. G., *Ann. Rev. Microbiol.*, **6**, 185 (1952)
5. McElroy, W. D., and Glass, B. H., Eds., *Symposium on Inorg. Nitrogen Metabolism* (Johns Hopkins Press, Baltimore 18, Md., 728 pp., 1956)
6. DeTurk, W. E., *Federation Proc.*, **16**, 292 (1957)
7. Farkas-Himsley, H., and Artman, M., *J. Bacteriol.*, **74**, 690 (1957)
8. Macmillan, A., *Physiol. Plantarum*, **9**, 470 (1956)
9. Britten, R., after Gale, E. F., *Symposia Soc. Exptl. Biol.*, **8**, 242 (1953)
10. Cowie, D. B., and Roberts, R. B., *J. Cellular Comp. Physiol.*, **44**, 327 (1954)
11. Morton, A. G., and Macmillan, A., *J. Exptl. Botany*, **5**, 232 (1954)

12. Morton, A. G., *J. Exptl. Botany*, **7**, 297 (1956)
13. Nason, A., and Evans, H. J., *J. Biol. Chem.*, **202**, 655 (1953)
14. Wainwright, S. D., and Nevill, A., *J. Gen. Microbiol.*, **14**, 47 (1956)
15. Macmillan, A., *J. Exptl. Botony*, **7**, 113 (1956)
16. Apparao, A., *Experientia*, **12**, 215 (1956)
17. Warner, A. C. I., *Biochem. J.*, **64**, 1 (1956)
18. Pringsheim, E. G., *Pure Cultures of Algae* (The Cambridge University Press, Cambridge, Eng., 119 pp., 1946)
19. Myers, J., *Ann. Rev. Microbiol.*, **5**, 157 (1951)
20. Fogg, G. E., *The Metabolism of Algae* (Methuen & Co., Ltd., London, Engl., 149 pp., 1953)
21. Proctor, V. W., *Am. J. Botany*, **44**, 141 (1957)
22. Syrett, P. J., *Physiol. Plantarum*, **9**, 19 (1956)
23. Syrett, P. J., *Physiol. Plantarum*, **9**, 28 (1956)
24. Syrett, P. J., *Physiol. Plantarum*, **9**, 165 (1956)
25. Syrett, P. J., *Physiol. Plantarum*, **9**, 924 (1955)
26. Macmillan, A., *Physiol. Plantarum*, **9**, 533 (1956)
27. Morton, A. G., and Broadbent, D., *J. Gen. Microbiol.*, **12**, 248 (1955)
28. Dulaney, E. L., Bilinski, E., and McConnell, W. B., *Can. J. Biochem. and Physiol.*, **34**, 1195 (1956)
29. Hall, L. M., and MacVicar, R., *J. Biol. Chem.*, **213**, 305 (1955)
30. Burma, D. P., and Burris, R. H., *J. Biol. Chem.*, **225**, 723 (1957)
31. Sato, R., *Sci. Rev.* (*Holland*), **2**, 122 (1950)
32. Kluyver, A. J., *Symposium Microbial Metabolism*, 771 (Intern. Congress of Microbiology, *6th Congr.*, Charles C Thomas, Publisher, Springfield, Ill., 144 pp., 1953)
33. Verhoeven, W., *Symposium on Inorg. Nitrogen Metabolism*, 61 (McElroy, W. D., and Glass, B. H., Eds., Johns Hopkins Press., Baltimore 18, Md., 728 pp., 1956)
34. Sato, R., *Symposium on Inorg. Nitrogen Metabolism*, 198 (McElroy, W. D., and Glass, B. H., Eds., Johns Hopkins Press, Baltimore, Md., 728 pp., 1956)
35. Sato, R., *Symposium on Inorg. Nitrogen Metabolism*, 163 (McElroy, W. D., and Glass, B. H., Eds., Johns Hopkins Press, Baltimore, Md., 728 pp., 1956)
36. Taniguchi, S., Sato, R., and Egami, F., *Symposium on Inorg. Nitrogen Metabolism*, 871 (McElroy, W. D., and Glass, B. H., Eds., Johns Hopkins Press, Baltimore, Md., 728 pp., 1956)
37. Sato, R., and Egami, F., *Bull. Chem. Soc. Japan*, **22**, 137 (1949)
38. Sato, R., and Niwa, M., *Bull. Chem. Soc. Japan*, **25**, 202 (1952)
39. Takahashi, H., Taniguchi, S., and Egami, F., *J. Biochem.* (*Tokyo*), **43**, 223 (1957)
40. Asnis, R. E., Vely, V. G., and Glick, M. C., *J. Bacteriol.*, **72**, 314 (1956)
41. Erkama, J., Häggman, J., and Wahlroos, Ö., *Ann. Acad. Sci. Fennicae*, [*A*] II, No. 60, 460 (1955)
42. Nicholas, D. J. D., and Nason, A., *J. Bacteriol.*, **69**, 580 (1955)
43. Evans, H. J., and Nason, A., *Plant Physiol.*, **28**, 233 (1953)
44. Nicholas, D. J. D., and Nason, A., *J. Biol. Chem.*, **207**, 353 (1954)
45. Nicholas, D. J. D., and Nason, A., *J. Biol. Chem.*, **211**, 183 (1954)
46. Nicholas, D. J. D., and Nason, A., *Plant Physiol.*, **30**, 135 (1955)
47. Wainwright, S. D., *Biochim. et Biophys. Acta*, **18**, 583 (1955)

48. Lascelles, J., *J. Gen. Microbiol.*, **15**, 404 (1956)
49. Granick, S., and Gilder, H., *J. Gen. Physiol.*, **30**, 1 (1946)
50. Sadana, J. C., and McElroy, W. D., *Arch. Biochem. Biophys.*, **67**, 16 (1957)
51. Gayon, V., and Dupetit, G., *Mem. soc. sci. phys. nat. Bordeaux* [3]**2**, 201 (1886)
52. Delwiche, C. C., *Symposium on Inorg. Nitrogen Metabolism*, 233 (McElroy, W. D., and Glass, B. H., Eds., Johns Hopkins Press, Baltimore, Md., 728 pp., 1956)
53. Verhoeven, W., and Takeda, Y., *Symposium on Inorg. Nitrogen Metabolism*, 159 (McElroy, W. D., and Glass, B. H., Eds., Johns Hopkins Press, Baltimore, Md., 728 pp., 1956)
54. Najjar, V. A., and Chung, C. W., *Symposium on Inorg. Nitrogen Metabolism*, 260 (McElroy, W. D., and Glass, B. H., Eds., Johns Hopkins Press, Baltimore, Md., 728 pp., 1956)
55. Sreenivasan, A., and Venkataraman, R., *J. Gen. Microbiol.*, **15**, 241 (1956)
56. Harvey, H. W., *Recent Advances in The Chemistry and Biology of Sea Water* (Cambridge University Press, Cambridge, Engl., 1945)
57. Kluyver, A. J., and Verhoeven, W., *Antonie van Leeuwenhoek. J. Microbiol. Serol.*, **20**, 2241, 337 (1954)
58. Beijerinck, M. W., *Arch. sci. exactes nat., Haarles* [2]**9**, 131 (1904)
59. Lieske, R., *Ber. deut. botan. Ges.*, **36**, 12 (1912)
60. Sacks, L. E., and Barker, H. A., *J. Bacteriol.*, **64**, 247 (1952)
61. Verhoeven, W., and Goos, J. J. C., *Antonie van Leeuwenhoek. J. Microbiol. Serol.*, **20**, 93 (1954)
62. Hansen, R. W., and Kallio, R. E., *Science*, **125**, 1198 (1957)
63. Stanier, R. Y., *Bacteriol. Rev.*, **14**, 179 (1950)
64. Hayaishi, O., Katagiri, M., and Rothberg, S., *J. Am. Chem. Soc.*, **77**, 5450 (1955)
65. Sacks, L. E., and Barker, H. A., *J. Bacteriol.*, **58**, 11 (1949)
66. Wijler, J., and Delwiche, C. C., *Plant and Soil*, **5**, 2 (1954)
67. Verhoeven, W., *Aerobic Sporeforming Nitrate Reducing Bacteria* (Doctoral thesis, Delft, The Netherlands, 1952)
68. Skerman, V. B. D., and MacRae, I. C., *Can. J. Microbiol.*, **3**, 215, 505 (1957)
69. Collins, F. M., *Nature*, **175**, 173 (1955)
70. Fedorov, M. V., and Sergeeva, R. V., *Mikrobiologiya*, **26**, 151 (1957)
71. Kefauver, M., and Allison, F. E., *J. Bacteriol.*, **73**, 8 (1957)
72. Sacks, L. E., *Metabolic Studies on Denitrification by* Pseudomonas denitrificans (Doctoral thesis, Univ. of California, Berkeley, Calif., 1948)
73. Allen, M. B., and Van Niel, C. B., *J. Bacteriol.*, **64**, 397 (1952)
74. Baalsrud, K., and Baalsrud, K. S., *Arch. Mikrobiol.*, **20**, 34 (1954)
75. Kamen, M. D., and Vernon, L. P., *Biochim. et Biophys. Acta*, **17**, 10 (1955)
76. Postgate, J., *Biochem. J.*, **56**, XI (1954); **58**, IX (1954)
77. Vernon, L. P., *J. Biol. Chem.*, **222**, 1035 (1956)
78. Vernon, L. P., *J. Biol. Chem.*, **222**, 1045 (1956)
79. Lenhoff, H. M., Nicholas, D. J. D., and Kaplan, N. O., *J. Biol. Chem.*, **220**, 983 (1956)
80. Nason, A., *Symposium on Inorg. Nitrogen Metabolism*, 109 (McElroy, W. D., and Glass, B. H., Eds., Johns Hopkins Press, Baltimore, Md., 728 pp., 1956)
81. Chung, C. W., and Najjar, V. A., *J. Biol. Chem.*, **218**, 617, 627 (1956)

82. Allen, M. B., and Van Niel, C. B., *J. Bacteriol.*, **64,** 397 (1952)
83. Kluyver, A. J., and Verhoeven, W., *Antonie van Leeuwenhoek J. Microbiol. Serol.*, **20,** 241 (1954)
84. Najjar, V. A., and Allen, M. B., *J. Biol. Chem.*, **206,** 209 (1954)
85. Spencer, D., Takahashi, H., and Nason, A., *J. Bacteriol.*, **73,** 553 (1957)
86. Yamada, T., and Virtanen, A. I., *Acta Chem. Scand.*, **10,** 20 (1956)
87. Iwasaki, H., Matsubayashi, R., and Mori, T., *J. Biochem.* (*Tokyo*), **43,** 295 (1956)
88. Iwasaki, H,. and Mori, T., *J. Biochem.* (*Tokyo*), **42,** 375 (1957)
89. Pollock, M. R., *Brit. J. Exptl. Pathol.*, **27,** 419 (1946)
90. Wainwright, S. D., and Nevill, A., *J. Bacteriol.*, **71,** 254 (1956)
91. Straughn, W. R., *Bacteriol. Proc.* (*Soc. Am. Bacteriologists*), 119 (1955)
92. Evans, H. J., and Hall, N. S., *Science,* **122,** 922 (1955)
93. Evans, H. J., *Plant Physiol.*, **29,** 298 (1954)
94. Cheniae, G. M., and Evans, H. J., *Plant Physiol.*, **31,** Suppl. X (1956)
95. Cheniae, G. M., and Evans, H. J., *Biochim. et Biophys. Acta,* **26,** 654 (1957)
96. Nicholas, D. J. D., *J. Sci. Food Agr.*, **8**(s) 15 (1957)
97. Nicholas, D. J. D., and Stevens, H. M., *Symposium on Inorg. Nitrogen Metabolism,* 178 (McElroy, W. D., and Glass, B. H., Eds., Johns Hopkins Press, Baltimore, Md., 728 pp., 1956); *Nature,* **176,** 1066 (1955)
98. McElroy, W. D., *Symposium on Inorg. Nitrogen Metabolism,* 573 (McElroy, W. D., and Glass, B. H., Eds., Johns Hopkins Press, Baltimore, Md., 728 pp., 1956)
99. Nicholas, D. J. D., and Scawin, J. H., *Nature,* **178,** 1474 (1956)
100. Kinsky, S. C., and McElroy, W. D., *Arch. Biochem. Biophys.* **73,** 466 (1958)
101. Stoy, V., *Plant Physiol.*, **8,** 963 (1955)
102. Stoy, V., *Biochim. et Biophys. Acta,* **21,** 395 (1956)
103. Egami, F., Ohmachi, K., Lida, K., and Taniguchi, S., *Biokhimiya,* **22,** 122 (1957)
104. Candela, M. I., Fisher, E. G., and Hewitt, E. J., *Plant Physiol.,* **32,** 280 (1957)
105. Tang, P., and Hsiang, Y. W., *Nature,* **179,** 1355 (1957)
106. Silver, W. S., *J. Bacteriol.*, **73,** 241 (1957)
107. Costilow, R. N., and Humphreys, T. W., *Science,* **121,** 168 (1955)
108. Wolfe, M., *Ann. Botany* (*London*), **18,** 299 (1954)
109. Wolfe, M., *Ann. Botany* (*London*), **18,** 309 (1954)
110. Arnon, D. I., Ichioka, P. S., Wessel, G., Fujiwara, A., and Wooley, J. T., *Physiol. Plantarum,* **8,** 538 (1955)
111. Ichioka, P. S., and Arnon, D. I., *Physiol. Plantarum,* **8,** 552 (1955)
112. Higgins, E. S., Richert, D. A., and Westerfeld, W. W., *Proc. Soc. Exptl. Biol. Med.*, **92,** 509 (1956)
113. Takahashi, H., and Nason, A., *Biochim. et Biophys. Acta,* **23,** 433 (1957)
114. Nicholas, D. J. D., *J. Gen. Microbiol.*, **15,** 470 (1956)
115. Omura, H., *J. Fac. Agr. Kyushu Univ.*, **10,** 365 (1956); *Chem. Abstr.*, **51,** 1344b (1957)
116. Hattori, A., *J. Biochem.* (*Tokyo*), **44,** 253 (1957)
117. Barnett, A. J. G., and Brown, I. B. R., *Biochem. J.*, **63,** 15p (1956)
118. Yamagata, S., *Acta Phytochim.* (*Japan*), **11,** 145 (1939–40)
119. Zucker, M., and Nason, A., *J. Biol. Chem.*, **213,** 463 (1955)

120. Nason, A., Abraham, R. G., and Averbach, B. C., *Biochim. et Biophys. Acta*, **15**, 159 (1954)
121. Medina, A., and Nicholas, D. J. D., *Nature*, **179**, 533 (1957)
122. McNall, E. G., and Atkinson, D. E., *J. Bacteriol.*, **72**, 226 (1956)
123. Atkinson, D. E., *J. Bacteriol.*, **71**, 78 (1955)
124. Krasna, A. I., and Rittenberg, D., *Proc. Natl. Acad. Sci. U.S.*, **40**, 225 (1954)
125. Keilin, J., *Biochem. J.*, **59**, 571 (1955)
126. McNall, E. G., and Atkinson, D. E., *J. Bacteriol.*, **74**, 60 (1957)
127. Senez, J. C., Pichinoty, F., and Konovaltchikoff-Mazoyer, M., *Compt. rend.*, **242**, 570 (1956)
128. Pichinoty, F., and Senez, J. C., *Compt. rend. soc. biol.*, **150**, 744 (1956)
129. Krasna, A. I., and Rittenberg, D., *Proc. Natl. Acad. Sci. U.S.*, **42**, 180 (1956)
130. Wickerham, L. J., *J. Bacteriol.*, **74**, 832 (1957)
131. Virtanen, A. I., and Saris, N. E., *Acta Chem. Scand.*, **9**, 337 (1955); **10**, 483 (1956)
131a. Seaman, G. R., *Biochim. et Biophys. Acta*, **26**, 313 (1957)
132. Fedorov, M. V., and Ilina, T. K., *Mikrobiologiya*, **25**, 537 (1956); *Chem. Abstr.*, **51**, 8888f (1957)
133. Kessler, E., *Planta*, **45**, 94 (1955)
134. Kessler, E., *Arch. Biochem. Biophys.*, **62**, 241 (1956)
135. Kessler, E., *Planta*, **49**, 505 (1957)
136. Vanecko, S., and Varner, J. E., *Plant Physiol.*, **30**, 388 (1955)
137. Medina, A., and Nicholas, D. J. D., *Biochim. et Biophys. Acta*, **23**, 440 (1957)
138. Evans, H. J., and McAuliffe, C., *Symposium on Inorg. Nitrogen Metabolism*, 189 (McElroy, W. D., and Glass, B. H., Eds., Johns Hopkins Press, Baltimore, Md., 728 pp., 1956)
139. Chung, C. W., *Symposium on Inorg. Nitrogen Metabolism*, 281 (McElroy, W. D., and Glass, B. H., Eds., Johns Hopkins Press, Baltimore, Md., 728 pp., 1956)
140. Nason, A., *Symposium on Inorg. Nitrogen Metabolism*, 289 (McElroy, W. D., and Glass, B. H., Eds., Johns Hopkins Press, Baltimore, Md., 728 pp., 1956)
141. Garner, G. B., Baumstark, J. S., Muhrer, M. E., and Pfander, W. N., *Anal. Chem.*, **28**, 1589–91 (1956)
142. Nicholas, D. J. D., and Nason, A., *Methods in Enzymology*, **III**, 981 (Colowick, S. P., and Kaplan, N. O., Eds., Academic Press, Inc., New York, N.Y., 1154 pp., 1957)
143. Medina, A., and Nicholas, D. J. D., *J. Gen. Microbiol.*, **17**, V (1957)
144. Medina, A., and Nicholas, D. J. D., *Biochim. et Biophys. Acta*, **25**, 138 (1957)
145. Bulen, W. A., *Plant Physiol.*, **31**, S XXIX (1956)
146. Vanecko, S., and Frear, D. S., *Plant Physiol.*, **30**, XXVI (1955)
147. Klausmeier, R., and Bard, R., *J. Bacteriol.*, **68**, 129 (1954)
148. Roussos, G. G., Takahashi, H., and Nason, A., *J. Bacteriol.*, **73**, 594 (1957)
149. Kono, M., Taniguchi, S., and Egami, F., *J. Biochem. (Tokyo)*, **44**, 615 (1957)
150. Ishimoto, M., Yagi, T., and Shiraki, M., *J. Biochem. (Tokyo)*, **44**, 707 (1957)
151. McElroy, W. D., and Spencer, D., *Symposium on Inorg. Nitrogen Metabolism*, 137 (McElroy, W. D., and Glass, B. H., Eds., Johns Hopkins Press, Baltimore, Md., 728 pp., 1956)

152. Silver, W. S., and McElroy, W. D., *Arch. Biochem. Biophys.*, **51**, 379 (1954)
153. Saz, A. K., Brownell, L. W., and Slie, R. B., *J. Bacteriol.*, **71**, 421 (1956)
154. Saz, A. K., and Martinez, M., *J. Biol. Chem.*, **223**, 285 (1956)
155. Saz, A. K., and Martinez, M., *Bacteriol. Proc. (Am. Soc. Bacteriologists)*, 114 (1956)
156. Asnis, R. E., *Arch. Biochem. Biophys.*, **66**, 208 (1956)
157. Tsukamura, M., *Igaku to Seibutsugaku*, **33**, 59, 270 (1954); *Chem. Abstr.*, **50**, 10152b (1956)
158. Krasna, A. I., and Rittenberg, D., *J. Am. Chem. Soc.*, **77**, 5295 (1955)
159. Cormier, M. J., and Rostorfer, H. H., *Bacteriol. Proc. (Am. Soc. Bacteriologists)*, 114 (1956)
160. Kiese, M., Resag, K., and Schneider, C., *Arch. exptl. Pathol. Pharmakol., Naunyn-Schmeideberg's*, **231**, 170, 176 (1957); *Chem. Abstr.*, **51**, 18039b (1957)
161. Westerfeld, W. W., Richert, D. A., and Higgins, E. S., *J. Biol. Chem.*, **227**, 379 (1957)
162. Jensen, H. L., and Gundersen, K., *Nature*, **175**, 341 (1955)
163. Little, H. N., *J. Biol. Chem.*, **229**, 231 (1957)
164. Delwiche, C. C., *Symposium on Inorg. Nitrogen Metabolism*, 218 (McElroy W. D., and Glass, B., Eds., Johns Hopkins Press, Baltimore, Md., 728 pp., 1956)
165. Fisher, T., Fisher, E., and Appleman, M. D., *J. Gen. Microbiol.*, **14**, 238 (1956)
166. Ruban, E. L., *Mikrobiologiya*, **25**, 150 (1956); *Chem. Abstr.*, **50**, 12186D (1956)
167. Alleem, M. I. H., Engel, M. S., and Alexander, M., *Bacteriol. Proc. (Am. Soc. Bacteriologists)*, 9 (1957)
168. Klein., R., *Nature*, **179**, 1200 (1957)
169. Gundersen, K., *Nature*, **179**, 789 (1957)
170. Zavarzin, G. A., *Doklady Akad. Nauk S. S. S. R.*, **113**, 1361 (1957); *Chem. Abstr.*, **51**, 16693a (1957)
171. Hoffman, T., and Lees, H., *Biochem. J.*, **54**, 579 (1953)
172. Lees, H., and Simpson, J. R., *Biochem. J.*, **65**, 297 (1957)
173. Imshenetskii, A. A., Ruban, E. L., and Buzina, O. D., *Mikrobiologiya*, **24**, 539 (1955); *Chem. Abstr.*, **49**, 12604h (1956)
174. Imshenetskii, A. A., Ruban, E. L., and Artemova, L. I., *Mikrobiologiya*, **25**, 1245 (1956); *Chem. Abstr.*, **50**, 12146b (1956)
175. Imshenetskii, A. A., and Ruban, E. L., *Folia Biol.* (Prague), **3**, 141 (1957); *Chem. Abstr.*, **51**, 1672og (1957)
176. Ware, G. C., and Painter, H. A., *Nature*, **175**, 900 (1955)
177. Gest, H., Judis, J., and Peck, H. D., *Symposium on Inorg. Nitrogen Metabolism*, 298 (McElroy, W. D., and Glass, B. H., Eds., Johns Hopkins Press, Baltimore, Md., 728 pp., 1956)
178. Burris, R. H., *Symposium on Inorg. Nitrogen Metabolism*, 316 (McElroy, W. D., and Glass, B. H., Eds., Johns Hopkins Press, Baltimore, Md., 728 pp., 1956)
179. Shug, A. L., Hamilton, P. B., and Wilson, P. W., *Symposium on Inorg. Nitrogen Metabolism*, 344 (McElroy, W. D., Glass, B. H., Eds., Johns Hopkins Press, Baltimore, Md., 728 pp., 1956)
180. Hamilton, P. B., Shug, A. L., and Wilson, P. W., *Proc. Natl. Acad. Sci. U.S.*, **43**, 297 (1957)
181. Hoch, G. E., Little, H. N., and Burris, R. H., *Nature*, **179**, 430 (1957)

182. Lee, S. B., and Wilson, P. W., *J. Biol. Chem.*, **151,** 377 (1943)
183. Hiai, S., Mori, Takeshi, Hino, S., and Mori, Takako, *J. Biochem. (Tokyo)*, **44,** 839 (1957)
184. Rosenblum, E. D., and Wilson, P. W., *J. Bacteriol.*, **59,** 83 (1950)
185. Winfield, M. E., *Rev. Pure and Appl. Chem.*, **5,** 21 7(1955)
186. Nason, A., Takahashi, H., Hoch, G. E., and Burris, R. H. *Federation Proc.*, **16,** 224 (1957)
187. Jose, A. G., and Pengra, R. M., *Bacteriol. Proc. (Am. Soc. Bacteriologists)*, 132 (1957)
188. Allison, R. M., and Burris, R. H., *J. Biol. Chem.*, **224,** 351 (1957)
189. Burma, D. P., and Burris, R. H., *J. Biol. Chem.*, **225,** 287 (1957)
190. Mozen, M. M., and Burris, R. H., *J. Bacteriol.*, **70,** 127 (1955)
190a. Saris, N., and Virtanen, A. I., *Acta Chem. Scand.*, **11,** 1438 (1957)
190b. Saris, N., and Virtanen, A. I., *Acta Chem. Scand.*, **11,** 1440 (1957)
191. Bach, M. K., and Burris, R. H., *Federation Proc.*, **16,** 148 (1957)
192. Bach, M. K., *Biochim. et Biophys. Acta*, **26,** 104 (1957)
193. Azim, M. A., and Roberts, E. R., *Biochim. et Biophys. Acad.*, **21,** 563 (1956)
194. Burris, R. H., Magee, W. E., and Bach, M. K., *Ann. Acad. Sci. Fennicae* [A] II, No. 60, 190 (1955)
195. Rakestraw, J. A., and Roberts, E. R., *Biochim. et Biophys. Acta*, **24,** 388 (1957)
196. Rakestraw, J. A., and Roberts, E. R., *Biochim. et Biophys. Acta*, **24,** 555 (1957)
197. Azim, M. A., and Roberts, E. R., *Biochim. et Biophys. Acta*, **21,** 308 (1956)
198. Hino, S., *J. Biochem. (Tokyo)*, **42,** 775 (1955)
199. Fedorov, M. V., and Kalininskaya, T. A., *Mikrobiologiya*, **26,** 3 (1957)
200. Bullen, W. A., and Frear, D. S., *Arch. Biochem. Biophys.*, **66,** 502 (1957)
201. Bové, J. Bové, C., and Arnon, D. I., *Plant Physiol.*, **32,** Suppl. XXIII (1957)
202. Magee, W. E., and Burris, R. H., *J. Bacteriol.*, **71,** 635 (1956)
203. Keeler, R. F., Bulen, W. A., and Varner, J. E., *J. Bacteriol.*, **72,** 394 (1956)
204. Keeler, R. F., *J. Bacteriol.*, **73,** 582 (1957)
205. Keeler, R. F., and Varner, J. E., *Arch. Biochem. Biophys.*, **70,** 585 (1957)
206. Keeler, R. F., and Varner, J. E., *Bacteriol. Proc. (Am. Soc. Bacteriologists)*, 122 (1957)
207. Esposito, R. G., and Wilson, P. W., *Proc. Soc. Exptl. Biol. Med.*, **93,** 564 (1956)
208. Allen, M. B., *Sci. Monthly*, **83,** 100 (1956)
209. Davies, E. B., and Stockdill, S. M. J., *Nature*, **178,** 866 (1956)
210. Allen, M. B., and Arnon, D. I., *Plant Physiol.*, **30,** 366 (1955)
211. Esposito, R. G., and Wilson, P. W., *Biochim. et Biophys. Acta*, **22,** 186 (1956)
212. Esposito, R. G., *Bacteriol. Proc. (Am. Soc. Bacteriologists)*, 113 (1957)
213. Wyss, O., and Chu, M. Y. W., *Federation Proc.*, **15,** 389 (1956)
214. Keeler, R. F., and Varner, J. E., *Nature*, **181,** 127 (1958)
215. Arnon, D. I., and Allen, M. B., *Physiol. Plantarum*, **8,** 653 (1955)
216. Tissières, A., and Burris, R. H., *Biochim. et Biophys. Acta*, **20,** 436 (1956)
217. Tissières, A., *Biochem. J.*, **64,** 582 (1956)
218. Wilson, T. G. G., and Wilson, P. W., *J. Bacteriol.*, **70,** 30 (1955)
219. Alexander, M., and Wilson, P. W., *J. Bacteriol.*, **71,** 252 (1956)
220. Bruemmer, J. H., Wilson, P. W., Glenn, J. L., and Crane, F. L., *J. Bacteriol.*, **73,** 113 (1957)
221. Layne, E. C., and Nason, A., *J. Biol. Chem.*, **231,** 889 (1958)
222. Thorogood, E., *Science*, **126,** 1011 (1957)

223. Rose, I. A., and Ochoa, S., *J. Biol. Chem.*, **220**, 307 (1956)
224. Tissières, A., Hovenkamp, H. G., and Slater, E. C., *Biochim. et Biophys. Acta*, **25**, 336 (1957)
225. Josten, J. J., *Bacteriol. Proc. (Am. Soc. Bacteriologists)*, 109 (1957)
226. Hartman, P. E., Brodie, A. F., and Gray, C. T., *J. Bacteriol.*, **74**, 319 (1957)
227. Bonnier, C., and Sironval, C., *Nature*, **177**, 93 (1957)
228. Monsour, V., and Wyss, O., *Federation Proc.*, **14**, 257 (1955)
229. Brown, M. E., and Metcalfe, G., *Nature*, **180**, 282 (1957)
230. Hamilton, P. B., and Wilson, P. W., *Ann. Acad. Sci. Fennicae* [A] II No. 60, 139 (1955)
231. Jensen, V., *Physiol. Plantarum*, **9**, 130 (1956)
232. Anderson, G. R., *J. Bacteriol.*, **70**, 129 (1955)
233. Rakhno, P. K., and Tokhuer, V. I., *Doklady Akad. Nauk. S. S. S. R.*, **112**, 144 (1957); *Chem. Abstr.*, **51**, 10652i (1957)
234. Roy, A. B., and Mukherjee, M. K., *Nature*, **180**, 236 (1957)
235. Kluyver, A. J., and Becking, J. H., *Ann. Acad. Sci. Fennicae* [A] II, No. 60, 367 (1955)
236. Ruinen, J., *Nature*, **177**, 220 (1956)
237. Watanabe, A., Nishigaki, S., and Konishi, C., *Nature*, **168**, 748 (1951)
238. Kratz, W. A., and Myers, J., *Am. J. Botany*, **42**, 282 (1955)
239. Bond, G., and Scott, G. D., *Ann Botany (London)*, **19**, 67 (1955)
240. Bond, G., *New Phytologist*, **55**, 147 (1956 )
241. Virtanen, A. I., *Physiol. Plantarum*, **10**, 164 (1957)
242. Bond, G., and Gardner, I. C., *Nature*, **179**, 680 (1957)
243. Bond, G., *J. Exptl. Botany*, **6**, 303 (1955)
244. Bond, G., MacConnell, J. T., and McCallum, A. H., *Ann. Botany (London)*, **20**, 501 (1956)
245. Khudairi, A. K., *Science*, **125**, 399 (1957)
246. Parker, C. A., *Nature*, **179**, 593 (1957)
247. Heppel, L. A., and Porterfield, V. T., *J. Biol. Chem.*, **178**, 549 (1949)

# MICROBIAL NUTRITION[1]

By Beverly M. Guirard[2]
*Department of Biochemistry, University of California, Berkeley, California*

The field of microbial nutrition has gradually evolved from one primarily concerned with the identification of factors required for growth in defined media to one in which more emphasis is directed toward elucidating biosynthetic pathways of cell constituents and products, control mechanisms for regulating growth, the functional role of biochemical catalysts, and the nature of cell permeability and its effect on cellular activities.

Probably the most significant contribution of the past two or three years to the field of microbiology was the demonstration that the entrance of certain organic substances into microbial cells was not a passive phenomenon but instead involved specific transport systems. The nature of these permeation systems (permeases) and evidence of their occurrence have recently been reviewed by Cohen & Monod (1). The energy relationships involved in membrane transport are discussed by Mitchell (2).

The site of the transport mechanisms is thought to be in the cell membranes; however, in some cases it may be in membranes surrounding subunits of the cell (3). Rickenberg (4) found that intact cells and protoplasts of a strain of *Escherichia coli* incorporated lactose-1-$C^{14}$ to approximately the same extent. This finding indicated that the same mechanism for galactoside accumulation could be operating in the intact bacterium as in the bacterium deprived of its cell wall, and that the protoplast membrane, not the cell wall, was the site of galactoside-permease activity.

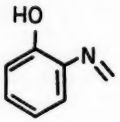
Engelhard *et al.* (5) studied the effects of treating a virulent strain of *Mycobacterium tuberculosis* with trypsin. Permeability alterations were observed which were reflected in the decreased time required to reduce methylene blue, the increased capacity to oxidize certain substrates, alteration in staining properties, and the change in cultural requirements.

The numerous studies of Gale and his associates on the assimilation of amino acids by bacteria have contributed much to our knowledge of membrane transport. Their recent study on the accumulation of free glutamic acid in cells of *Staphylococcus aureus* (6) reveals that glutamic acid must pass some barrier in entering the cell since certain glutamyl peptides permit a more rapid accumulation of glutamic acid within the cell than glutamic acid itself. The rate of accumulation of glutamic acid is higher with α-glutamyl than with γ-glutamyl peptides. The accumulation did not take place in the absence of glucose and was inhibited by sodium azide and

[1] The survey of the literature pertaining to this review was concluded in January, 1958.

[2] The author is grateful to Dr. Esmond E. Snell for reading and criticizing the manuscript.

2,4-dinitrophenol. Gale & Folkes (7) also studied the incorporation of glycine-$C^{14}$ by disrupted staphylococcal cells in the presence of an energy source. They found that the incorporation of glycine was inhibited by 8-hydroxyquinoline in media containing heavy metals and by various benzimidazole derivatives. 6-Amino-4-hydroxy derivatives of either benzimidazole or benzotriazole stimulated incorporation and antagonized the inhibitory action of other benzimidazol derivatives. The results were explained on the basis of the possibility of the following structure,




being an active center in the incorporation mechanism. The structure is present in many of the substances tested which affected incorporation. Also, the inhibition by 8-hydroxyquinoline chelates could be explained on the basis of competition between similar structures.

Since the subject of permeability and membrane transport pervades the entire content of this review, the relevant contributions of the past year have been discussed first. The selective capacity of bacterial cells to determine the substances which enter the cell and the rate at which they enter should be a fundamental consideration of all nutritional studies. The scope of this review will be limited to certain aspects of the nutrition of bacteria and fungi. The reviewer has found it difficult to delete any topic concerning growth and metabolism on the basis of impertinent data alone. The imposed delimits, then, serve to adjust the length of the review to the space allotted.

## Requirements for Growth and Biosynthesis of Cell Constituents

A well-defined growth medium for studying almost any type of microbial activity and faithful reporting of cultural conditions are well-recognized necessities. Studies of growth requirements are also valuable in determining conditions which favor high cell yields for enzyme fractionation, development of cultures with particular characteristics, maintaining pathogenic strains without loss of virulence, ascertaining factors responsible for initiating rapid growth from small inocula, and developing or improving microbiologic methods of assay.

*Carbon and energy sources.*—Bacteria of the genus *Pseudomonas,* many strains of *E. coli,* and many molds are capable of utilizing two-carbon compounds to satisfy their entire carbon requirement. Recently, Kornberg & Krebs (8) have described a modification of the tricarboxylic acid cycle operative in these organisms. The main discoveries which led to the formulation of the cycle were: (*a*) discovery of the reversible enzymatic cleavage

of isocitrate to succinate and glyoxylate (9, 10, 11); (*b*) the malate synthetase reaction discovered by Wong & Ajl (12) by which glyoxalate and acetyl coenzyme A react to form malate; (*c*) the demonstration that the two enzyme systems are readily coupled in cell-free extracts (13). The sequence of reactions is as follows: acetate + oxalacetate → citrate → isocitrate → succinate + glyoxalate; glyoxalate + acetate → malate; malate + ½ $O_2$ → oxalacetate.

The net effect of one turn of the cycle is, 2 acetate + ½ $O_2$ → succinate; whereas one turn of the tricarboxylic acid cycle yields, acetate + 2 $O_2$ → 2 $CO_2$ The steps between citrate and malate in the tricarboxylic acid cycle are replaced by reactions involving glyoxylate, the key intermediate from which the cycle derives its name. The glyoxylate cycle accounts for the net synthesis of $C_4$-dicarboxylic acids which can (*a*) serve to feed the tricarboxylic acid cycle with oxalacetate, or (*b*) permit utilization of acetate to form many cell constituents including hexoses, pentoses, carbon skeletons of several amino acids. It can also account for the formation of citric and fumaric acids in molds.

The energy sources utilized by *Vibrio fetus*, the organism responsible for abortion in cattle, have been studied by Alexander (14). Compounds which could be used as sources of energy were those which could also be oxidized by resting cell suspensions. These were: lactate, pyruvate, acetate, α-ketogutarate, succinate, fumarate, malate, aspartate, asparagine, glutamate, and proline. The organism appears to be totally lacking in fermentative ability.

The unusual ability of an anaerobic strain of *Lactobacillus bifidus* to utilize raffinose for growth was reported by Lloyd (15). The complete utilization of the trisaccharide was indicated by the fact that the yield of acid from this sugar was three times that derived from equimolar amounts of glucose. When the bacteria were treated with a detergent, acid production stopped and paper chromatography revealed the presence of galactose, sucrose, fructose, and glucose phosphates. Although intact cells were unable to utilize sucrose and fructose, detergent-treated cells degraded sucrose to glucose phosphates and glucose in the presence of phosphate, or into glucose and fructose in the presence of arsenate. These results indicated that sucrose and fructose were intermediates in the metabolism of raffinose.

A new strain of yeast was reported which also had the ability to ferment raffinose but not sucrose [Van Uden & Assis-Lopes (16)]. In the case of *Saccharomyces italicus* var. *melibiosi*, however, the galactose portion of the molecule was oxidized, leaving the sucrose portion intact. While the inability of *L. bifidus* to utilize sucrose and fructose might be explained on the basis of permeability barriers, the situation which obtains in the yeast culture appears to be different.

In studying the role of amino acid degradations as sources of energy for pleuropneumonia-like organisms, Smith has shown that adenosine triphosphate was generated by the deamination of glutamine (17) in the

presence of adenosine diphosphate and also by the phosphorolysis of citrulline (18). The latter reaction was considered to be of doubtful significance to these organisms, however, since the position of equilibrium of this reaction is in favor of citrulline synthesis.

The basic nutritional requirements of the following organisms have been determined: *Clostridium perfringens* by Fuchs & Bonde (19); *Micrococcus lysodeikticus* by Wolin & Naylor (20); *Pseudomonas fragi* by Pereira & Morgan (21); *Sapromyces elongatus, Rhipidium* sp. and *Apodachyla* sp. by Golueke (22); three strains of *Claviceps purpurea* by Taber & Vening (23); and eight strains of anaerobic streptococci by Mergenhagen & Scherp (24).

*Nitrogen sources.*—The reduction of nitrate to ammonia is thought to proceed through nitrite and hydroxylamine. The first step in the pathway has been well-established; but the reduction of nitrite to hydroxylamine has not been extensively studied and the intermediates not conclusively identified (25). The recent findings of McNall & Atkinson (25) provide support for the involvement of nitrite, hyponitrite or a closely related compound, and hydroxylamine as intermediates in nitrate reduction. *E. coli* strain Bn can utilize hyponitrite, hydoxylamine, or nitrous oxide as the sole source of nitrogen for growth as well as nitrate or nitrite. This organism appears to be the first known to grow on hydroxylamine as the sole source of nitrogen. The parent strain B, although unable to grow on hydroxylamine, did reduce it quantitatively to ammonia. Hydrazine did not support growth of either strain. In manometric experiments under an atmosphere of hydrogen, strain Bn, but not the parent strain, reduced nitrite, hyponitrite, and hydroxylamine to ammonia. Also, under anaerobic conditions, strain Bn, but not strain B, grew luxuriantly with lactate as a carbon source and nitrate as electron acceptor. The slow rate of adaptation to nitrous oxide in a liquid glucose-mineral salts medium lacking fixed nitrogen indicated to the authors that nitrous oxide is not an intermediate in nitrate reduction.

*Amino acids and peptides.*—The methionine requirement of *Streptococcus lactis* 829 was studied by Husain & McDonald (26). A medium containing seven amino acids (arginine, histidine, isoleucine, leucine, phenylalanine, proline, and valine), and other essential nutrients including pyridoxal, supported growth of the organism in the presence of methionine, but not in its absence. In this medium, methionine could be replaced by homocysteine or cystathionine but not by cysteine or other sulfur-containing compounds. Serine did not improve the extent of growth obtained with homocysteine. In a medium containing 18 amino acids, pyridoxal was not essential for growth. In the latter medium without pyridoxal and methionine, cystathionine did not support growth and homocysteine supported only 35 per cent of the growth obtained with methionine. When pyridoxal was included in the medium, the same amount of growth was obtained with

homocysteine, cystathionine, or methionine. The authors conclude that the data are not sufficient to propose a mechanism for synthesis of methionine from homocysteine, but do suggest that vitamin $B_6$ is involved in this conversion and that serine is not implicated. These results concerning serine are at variance with those obtained by Kizer *et al.* (*27*) with other strains of *S. lactis*. They concluded that methionine was synthesized from homocysteine through methylation by the β-carbon of serine.

Simmonds & Miller (*28*) have studied serine and glycine requirements of certain auxotrophs of *E. coli* strain K-12. The serine auxotroph, strain S, utilized glycine for growth, and the response to glycine was adaptive. Glycine peptides permitted more rapid growth of this strain than did glycine. Strain S/G, which responds equally well to serine and glycine, was able to convert serine into glycine. The main difference between the two strains appeared to be the deficiency in strain S of a mechanism for taking up glycine from the culture medium.

Because of conflicting reports in the literature regarding the nutrition of *Hemophilus (Bordetella) pertussis*, Jebb & Tomlinson (*29*) have reinvestigated the sulfur and minimal amino acid requirements of this organism. Of the 13 sulfur-containing compounds tested, only cystine, cysteine and, to a lesser extent, glutathione supported growth on a chemically defined medium. Using very small inocula, eight strains of this organism were able to grow on a medium containing only two amino acids, cystine plus glutamic acid.

The amino acid and vitamin requirements of several strains of *Clostridium histolyticum* (*30*), of 22 strains of *Bacillus coagulans* (*31*), and of some 180 strains of thermophilic bacteria (*32*) including *Bacillus stearothermophilus* as well as *Bacillus coagulans*) have been studied. Also, the effect of certain amino acids on growth of two varieties of the pathogenic fungus, *Ophiobolus graminis* (*33*), and the utilization of amino acids as carbon and nitrogen sources for a species of marine bacterium (*34*) have been reported.

The utilization of the D-isomers of the amino acids, the corresponding L-isomers of which are required for growth by certain lactobacilli, has been studied by Koser & Thomas (*35*). The L- or DL-forms of the amino acids were omitted singly from the culture medium and substituted by the corresponding D-forms. The occurrence of growth under these conditions was interpreted as utilization, although the involvement of antagonistic effects between amino acids and the dependance of utilization of one form on the presence of the other was not overlooked. *L. casei* strains did not grow on D-phenylalanine, D-valine, D-leucine, and D-isoleucine; however, they did grow in the presence of D-glutamic or D-aspartic acids, but at a slower rate than in the presence of the corresponding L-amino acids. *L. plantarum* did not respond to D-phenylalanine or D-aspartic acid and its response to D-isoleucine, D-glutamic, and D-leucine was delayed. Among the organisms

tested, *L. fermenti* was best able to utilize the D-amino acids, responding promptly to D-valine, D-leucine, D-isoleucine, D-glutamic acid, and D-aspartic acid. Its response to D-phenylalanine was slow or negligible. Different strains of each species behaved in a similar manner.

Evidence for the sequence of reactions occurring in *E. coli* by which anthranilic acid is converted to tryptophan has been furnished by the isotopic and enzymatic studies of Yanofsky and of Trudinger [see Yanofsky (36)]. Yanofsky (36) has now provided evidence for the participation of this series of reactions *in vivo* by demonstrating that the enzymes catalyzing these reactions are lacking in specific tryptophan auxotrophs. He has correlated the nutritional, metabolite accumulation, and enzymatic data on the 15 tryptophan auxotrophs studied, and concluded that the following sequence represents the main pathway of tryptophan biosynthesis in *E. coli:* Anthranilic acid + phosphoribosyl pyrophosphate → indole-3-glycerol phosphate → indole + triosephosphate; indole + L-serine → L-tryptophan. Previous studies have indicated that tryptophan is synthesized by the same pathway in *Neurospora crassa, Salmonella typhimurium,* and another strain of *E. coli.*

Parks & Douglas (37) have found that an auxotroph of *Saccharomyces,* capable of growing on either indole or tryptophan, secreted anthranilic acid and a fructose derivative into the growth medium. The fructose derivative was identified as N-fructosyl anthranilic acid. Its role as an intermediate in indole biosynthesis is discussed; it is suggested that the derivative probably occurs phosphorylated as proposed for intermediates in histidine biosynthesis (imidazole glycerol phosphate, imidazole acetol phosphate, histidinol phosphate) by histidine-requiring mutants of *Neurospora* (38).

*Vitamins.*—The presence of an oligo-glutamate form of the citrovorum factor (CF) in extracts of *Bacillus subtilis* has been reported by Hakala & Welch (39). Evidence for its identity as the triglutamate was obtained by comparing its properties with the CF derivative prepared enzymatically by incubating synthetic pteroyldiglutamyl glutamic acid (teropterin) with a cell-free sonic extract of *S. faecalis* (40). The two CF derivatives and their acid, alkali, and enzymatic degradation products were identical in their microbial activities and chromatographic properties.

The family of substances related to vitamin $B_{12}$ or capable of replacing vitamin $B_{12}$ for growth under restricted conditions continues to increase. Seaman & Sanders (41) have reported a material in cells of *Crithidia fasciculata* which replaces vitamin $B_{12}$ for growth of *E. coli* 113-3, the soil bacterium, "Lochhead 38," and *Euglena gracilis,* but not *Ochromonas malhamensis.* The inactivity of the "Crithidia factor" for the latter organism [known to be specific for cyanocobalamin (42)], and the absence of microbiologically active fragments when *Crithidia* extracts are subjected to alkaline degradation, have indicated to the authors that Factor B (the non-nucleotide portion of cobalamin) is not a component of this sub-

stance. They suggest the occurrence in nature of a new series of vitamin $B_{12}$-functioning materials which do not possess the cobalamin structure.

Barchielli *et al.* (43) have found two analogues of vitamin $B_{12}$ active for *E. coli* 113-3 in the fermentation broth of a strain of *Nocardia*. The two red substances were separated from vitamin $B_{12}$ and factor B, also found in the culture medium, and purified. No benzimidazole derivatives were found after hydrolysis by 6 *N* HCl. Hydrolysis by 2 *N* HCl liberated a purine whose ultraviolet spectrum and chromatographic behavior were similar to those of guanine. The factor present in larger amounts was deaminated by nitrous acid; the deaminated product upon acid hydrolysis yielded a purine indistinguishable from xanthine. The properties of the guanine-containing analogue of vitamin $B_{12}$ are closely similar to those of Factor C, described by Ford *et al.* (44). The other vitamin $B_{12}$-active substance was not affected by nitrous acid and behaved like vitamin $B_{12}$ in electrophoresis.

Ford & Hutner (45) have studied the nature of the vitamin $B_{12}$ requirement of 19 isolates of soil bacteria and have discussed the usefulness of these organisms as research tools. One of these soil isolates was studied in detail (46).

The biosynthesis of vitamin $B_{12}$ was studied in a strain of *Streptomyces griseus* by Krasna *et al.* (47). Vitamin $B_{12}$ isolated from the culture filtrate of organisms grown in the presence of L-threonine-$N^{15}$ had a high concentration of $N^{15}$ in the aminopropanol fragment. The probable formation of aminopropanol by decarboxylation of threonine is the first evidence of such a reaction occurring *in vivo* (47).

The synthesis of substances possessing vitamin $B_{12}$ activity was also studied in a strain of *Bacillus* and one of *Micrococcus* isolated from fresh clams (48). In addition to 5,6-dimethylbenzimidazolehydroxycobalamin produced by the *Bacillus*, a number of cobalamins were produced when 6-nitrobenzimidazole was added to the growing cultures of both organisms.

Although pantothenylcysteine has been suggested as an intermediate in the synthesis of pantethine from pantothenic acid and cysteine in some microbial (49) and animal systems (50), its function in lactic acid bacteria was not well established (51). Brown (51) has recently studied the role of pantothenylcysteine in the nutrition and metabolism of *Lactobacillus helveticus*. Pantetheine or pantethine is about 100 times more active than an equimolar amount of pantothenic acid; and pantothenylcysteine is only slightly active. However, when this culture was sensitized to pantothenic acid and to pantothenylcysteine by successive transfers in media containing progressively smaller amounts of pantothenic acid and pantothenylcysteine, respectively, two mutant strains were selected which could utilize the three compounds equally well. Resting cells of the parent strain were unable to convert pantothenylcysteine to pantetheine; however, acetone-dried cells were as efficient as the resting cells or acetone-dried

cells of the mutants in carrying out this reaction. The results indicated that the parent *L. helveticus* was a mixed culture of a predominant strain which could not easily assimilate pantothenic acid and pantothenylcysteine and a small component of mutant strains which could assimilate pantothenic acid, pantethine, and pantothenylcysteine with equal ease. The discovery of pantothenylcysteine decarboxylase in *L. helveticus* and *L. bulgaricus* indicated that pantothenylcysteine is an intermediate in coenzyme A synthesis by lactic acid bacteria.

The effect of varying components of the growth medium on synthesis of riboflavin by *Candida flareri* was studied by Goodwin & McEvoy (52). Generally, replacement of ammonium sulfate with amino acids improved growth but decreased flavinogenesis. The effect of iron in decreasing flavinogenesis (53) was confirmed. Xanthine and uric acid but not the pyrimidines supported growth and flavinogenesis.

Many carbohydrate derivatives have been isolated or synthesized which possess growth-promoting activity for *Lactobacillus bifidus*. All of these contain N-acetyl-D-glucosamine (54). Pope, Tomarelli & György have described the isolation and identification of another such substance produced by a strain of *Aspergillus terreus* while degrading chitin. The factor possessed greater activity than N-acetyl-D-glucosamine, also found in the broth, and was identified as ethyl-N-acetyl-β-D-glucosaminide.

The utilization of inositol during growth of *Schizosaccharomyces pombe* has been thoroughly studied by Yarbrough & Clark (55). Inositol was taken up rapidly during log phase growth and afterwards until the concentration in the medium was only two to three per cent of that originally present. A "bound" form, unavailable to this organism for growth, began to appear in the medium after the completion of the log phase and continued to increase as the culture aged. The bound inositol is thought to arise from cell autolysis, and differs from inositol within the cell, which is mostly bound to lipides (possibly in lecithin-like compounds) and is extractable with fat solvents. Cells undergoing autolysis liberated both free and bound inositol. Phytin-like compounds were not found.

Studies of Charalampous (56) on the biosynthesis of inositol have indicated that glucose is not the immediate precursor of inositol in yeast, but that smaller fragments derived from glucose condense to form the biologically active cyclitol.

Preliminary studies of the growth requirements of *Nocardia carollina* strain S by Martin & Batt (57) revealed that the extent of growth produced by yeast extract in an acetate-ammonium sulfate medium could be duplicated with thiamine alone, or pyrithiamine or the pyrimidine portion of the thiamine molecule, but not by the thiazole portion. Utilization of pyrithiamine was presumed to be due to the ability of the organism to degrade it and to use the released pyrimidine for synthesis of thiamine. If ammonium sulfate of the medium were replaced by aspartate, strain S could grow in

the absence of thiamine; and pyruvic and α-ketoglutaric acids accumulated. The explanation that aspartate stimulated synthesis of thiamine, seemed unlikely since cells grown under these conditions contained negligible amounts of thiamine and the accumulation of products of thiamine-dependent reactions indicated a thiamine deficiency. The fact that the organism grew on a thiamine-free acetate-aspartate medium, but not on a medium containing acetate, succinate, and ammonium sulfate, suggested that thiamine may be required for the utilization of ammonium ions. Support for this hypothesis was furnished by the observation that the rate of growth on the acetate-aspartate medium was not affected by the addition of either thiamine or ammonium sulfate; but their simultaneous addition resulted in a greatly increased rate of growth.

The finding of Markov & Saev (58) that benzyl penicillin and benzylpenicillic acid can substitute for thiamine as a growth factor for penicillin-resistant staphylococci is interesting. It is less surprising, however, when the chemical structures of the two compounds are compared (58). Thiamine is a thiazole and penicillin a thiazolidine derivative.

*Inorganic ions.*—In an attempt to clarify the question of whether or not the calcium requirement for growth of *Azotobacter* depended on the state of nitrogen furnished, Norris & Jensen (59) compared the calcium requirement of several strains of *Azotobacter* for growth with fixed nitrogen or with elemental nitrogen. Calcium was needed at approximately the same concentration under both conditions. Five strains of *A. agile* did not require calcium for growth but were stimulated by it. Strontium substituted efficiently for calcium in all cases where the latter was required. *A. vinelandii* has been reported to have an absolute requirement for molybdenum while fixing nitrogen (60).

The utilization of sulfur from several organic and inorganic compounds for growth and hydrogen sulfide production by *C. perfringens* was studied by Fuchs & Bonde (61). The sulfur requirement of three strains of *C. perfringens* was not satisfied by sulfate, sulfite, thiosulfate, or sulfide. Cystine, cysteine, or glutathione was required by all three strains and one required methionine or homocyst(e)ine in addition. Hydrogen sulfide was produced by growing cultures from sulfite, thiosulfate, cystine, cysteine, and glutathione.

An interesting requirement for growth of *Gallionella ferruginea* was reported by Kucera & Wolfe (62). An available source of reduced iron was necessary for continuous growth. This requirement was met by including ferrous sulfide in the liquid enrichment medium.

The inorganic ion requirements for growth of marine bacteria have been studied in considerable detail by MacLeod & Onofrey (63).

*Purines, pyrimidines, and their derivatives.*—A mutant of *E. coli* previously reported to require dihydrouracil for growth in minimal medium (64) has recently been shown to utilize β-ureidopropionic acid, β-alanine,

or pantothenic acid as well (65). Also, Slotnick & Weinfeld (65) have shown that dihydrouracil satisfied the growth requirements of two other *E. coli* mutants, previously characterized as requiring β-alanine or pantothenate. The relative molar activities of these compounds as growth factors indicated that the conversion of dihydrouracil to β-alanine, previously shown to take place in animal tissues (66), also occurs in *E. coli.* Uracil, orotic acid, and dihydroorotic acid did not support growth of these mutants, indicating that no significant conversion of these compounds to dihydrouracil takes place in these organisms. The failure of incorporation of dihydrouracil-2-$C^{14}$ into nucleic acid pyrimidines of *E. coli* strain D-2 or *E. coli* W provided evidence that dihydrouracil is not an intermediate in the biosynthesis of nucleic acid pyrimidines in *E. coli.*

Cohen & Barner (67) reported that 5-methyldeoxycytidine satisfied the pyrimidine requirement of a thymine-requiring mutant of *E. coli* and permitted a normal rate of growth. It is hydrolyzed *in vitro* by the pyrimidine nucleoside deaminase from *E. coli* B at approximately the same rate as deoxycytidine. The conversion of 5-methyldeoxycytidine to thymidine *in vivo* is inhibited by such natural substances as deoxycytidine, cytidine, and deoxyuridine, which fact suggested to the authors that the formation of these compounds may govern the availability of thymidine for deoxyribonucleic acid (DNA) synthesis.

Cohen and his associates in a series of papers have reported their findings on the biosynthesis of bacterial and virus pyrimidines (68, 69, 70). In a uracil-requiring strain of *E. coli,* both ribonucleic acid (RNA) and DNA cytosine as well as RNA uracil were formed exclusively from uracil supplied in the medium; uracil was also the source of the pyrimidine ring of thymine, and glucose the source of the methyl group (68). Methionine was not incorporated into bacterial or virus purines, nor did it serve as a precursor of the methyl group of thymine (68). The subsequent work of Cohen *et al.* has been concerned with identifying the immediate precursor of the methyl group of thymine and other intermediates in the biosynthesis of methyl pyrimidines. As a working hypothesis they proposed that hydroxymethylpyrimidines or their 5,6-dihydro-derivatives are intermediates in the formation of methylpyrimidines (68, 69). The inactivity of these compounds in satisfying the pyrimidine requirements of the *E. coli* mutants indicated that they are not intermediates in the formation of thymine (70).

To test the possibility that α-amino-*n*-butyric acid might be the source of the 3-carbon chain of the pyrimidine ring in *Neurospora,* a pyrimidine-deficient strain was grown in the presence of α-aminobutyric acid-3-$C^{14}$ [Herrmann & Fairley (71)]. Contrary to expectations, the isolated RNA pyrimidines had a much lower specific radioactivity than the original aminobutyric acid-3-$C^{14}$. α-Aminobutyric acid isolated from the mycelium possessed some radioactivity, but much less than the original supplied, indicating that considerable synthesis of this compound had taken place during

growth of the mold. In addition, significant labeling was found in isoleucine which is known to be synthesized from α-aminobutyric acid in *Neurospora* (72). Thus, in spite of the indicated synthesis of α-aminobutyric acid, this organism required an exogenous source of the amino acid for growth in the absence of pyrimidines. From these considerations it was concluded that α-aminobutyric acid is a precursor for pyrimidines in this strain of *Neurospora.*

The uracil requirement of some lactic acid bacteria is not replaceable by orotate or its precursors, (73) while other species can utilize both uracil and orotate (74). Crawford *et al.* (74), investigating the possibiltiy of a direct utilization of uracil versus a prior conversion to orotate, found evidence for a new pathway of pyrimidine nucleotide synthesis. It involved a direct condensation of uracil with phosphoribosylpyrophosphate to form uridine-5′-phosphate. They were also able to show that in the lactic acid bacteria studied, there was a direct correlation between the nutritional requirements of the organism for pyrimidines and the presence of enzymes capable of forming nucleotides from these pyrimidines.

The biosynthesis of purines in microorganisms was thoroughly covered by Magasanik (75) in last year's review which included his recent publications on the subject. However, the finding of Aaronson & Rodriguez (76) that 4-amino-5-imidazolecarboxamidine was more efficient in satisfying the purine requirementof *Gaffkya homari* than 4-amino-5-imidazolecarboxamide (AICA), should be mentioned. This indicates a possible role for the carboxamidine in purine biosynthesis.

Cowie & Bolton (77) have found that purines are utilized for nucleic acid synthesis via metabolic pools in the same way that amino acid pools are utilized for protein synthesis in *Candida utilis* (78). The yeast growing in the presence of fructose and $C^{14}$-labeled adenine or guanine incorporated $C^{14}$ rapidly at first into the trichloracetic (TCA)-soluble fraction until the exogenous supply of purine approached exhaustion. This fraction then lost its radioactivity to the TCA-precipitable fraction of the cell. These results suggested that exogenous purines were incorporated into a pool (pool A) before conversion into the nucleic acid fraction of the cells. The size of this pool was dependent upon the exogenous purine concentration. In the absence of added purines, a pool of purine nucleotides (pool B) was formed which obtained its carbon solely from the metabolism of fructose. The size of pool B was independent of the purine concentration of the medium. The nucleotide pool B is the site of conversion of adenine to guanine nucleotides and is the preferred source of the purines for nucleic acid synthesis.

Reports concerning the incorporation of "unnatural" purine and pyrimidine bases into nucleic acids continue to appear in the literature. The incorporation in some cases is quite extensive. Smith & Matthews (79) report that 8-azaguanine was incorporated in amounts up to 40 per cent of the ribonucleic acid guanine of *Bacillus cereus.* The 8-azaguanine was rap-

idly replaced by guanine when the cells were then cultured in the presence of guanine. Mandel & Markham (80) found that 8-azaguanine incorporation into the nucleic acids of *B. cereus* occurred at the expense of guanine; the guanylic acid content of the bacterial nucleic acid hydrolysate was selectively decreased by the approximate amount of azaguanylic acid synthesized. Škoda *et al.* (81) have isolated and obtained in crystalline form 6-azauracil riboside which accumulated in cultures of *E. coli* B growing in the presence of sub-bacteriostatic concentrations of 6-azauracil. Dunn & Smith (82) report that for significant incorporation of the 5-halogenated uracils into the deoxyribonucleic acids of *E. coli* it is necessary that the cells be dependent upon an external supply of thymine for growth. It was shown independently by Prusoff (83) and by Mandel (84) that there is no direct relationship between the incorporation of the unnatural purine or pyrimidine into nucleic acids and growth inhibition.

*Biosynthesis of proteins.*—As in the case of the incorporation of "unnatural" purines and pyrimidines into nucleic acids, certain amino acid analogues have been shown to be incorporated into proteins (85). With some exceptions (86) the presence of the analogue usually results in diminished growth, which may be interpreted as an inhibition of protein or enzyme synthesis or both. An amino acid analogue which could support exponential growth and active enzyme synthesis in the absence of the naturally occurring, corresponding amino acid was sought and found by Cowie & Cohen (87) in the selenium analogue of methionine. The incorporation of selenomethionine into proteins of a methionine-requiring mutant of *E. coli* (87, 88) is, in itself, an interesting finding and enhances the biologic importance of selenium. The attainment of exponential growth on selenomethionine in the complete absence of methionine demonstrates that the synthesis of all essential enzymes occurred (87). Also, β-galactosidase was formed under these conditions in an active state. Selenium cannot entirely replace sulfur for *E. coli;* the selenium analogue of glutathione was not synthesized and the analogue of cysteine was formed to a limited extent only. The ability of selenomethionine to replace methionine in enzymatic transmethylations has been demonstrated by Mudd & Cantoni (89).

In studies on proteinase biosynthesis in *Streptococcus liquifaciens,* Hartman *et al.* (90) found that supplementing the synthetic growth medium with adenine, uracil, pyridoxal, and riboflavin resulted in maximal proteinase synthesis. The addition of biotin, folic acid, or thiamine decreased proteinase synthesis.

Kornberg (91) observed that the incorporation of radioactivity into the proteins of a strain of *Pseudomonas* growing in the presence of $C^{14}$-labeled ammonium acetate was less when unlabeled bicarbonate was present than in its absence. It appeared that during short-term experiments, bicarbonate was the source of most of the carbon utilized for protein initially syn-

thesized. Two possible explanations were offered: (*a*) a $CO_2$-fixation reaction of unexpected magnitude was involved; or (*b*) the addition of bicarbonate increased the steady-state concentration of a compound(s) in ready equilibrium with $CO_2$ at an early stage in the pathway from acetate to protein. Experiments designed to evaluate the contribution of the $CO_2$-fixation reaction to protein synthesis indicated that it accounted for only 15 to 17 per cent of the carbon incorporated in the synthesis of protein during a period of 2½ hr. These organisms contain an enzyme which can form malate from pyruvate and $CO_2$; hence, it is likely that the addition of bicarbonate temporarily raised the steady-state concentration of malate or oxalacetate and the protein synthesized during short-term experiments was derived from a pool of $C_4$-compounds of low radioactivity (91).

The problem concerning the interdependency of protein and nucleic acid biosynthesis in microorganisms has not yet been resolved. Of the several investigations of this subject reported during the past year (92 to 96) the majority of them present evidence in favor of the obligatory coupling of the two biosynthetic processes.

*Biosynthesis of inducible enzymes and their control.*—The nutritional aspects of inducible enzyme synthesis is an interesting phase of biochemical investigations and one for which microorganisms furnish particularly valuable tools for study. The studies of Pardee (97) on the inducible mechanism for accumulation of melibiose in *E. coli* cells are a confirmation and extension of the work of Rickenberg *et al.* (98) on galactoside-permease. The data show that the galactoside-permease is different from α- and β-galactosidase since the enzymes can be independently induced. It does not appear to have α-β specificity, that is, its formation can be induced by either α- or β-galactosides.

Shilo & Stanier (99) showed that certain strains of *Pseudomonas* isolated from soil by the elective culture technique utilized tartaric acids by means of specific inducible dehydrases. Most strains attacked only the isomer(s) upon which they had been cultured. In the presence of limiting concentrations of the substrate-inducer, the organism rapidly lost its ability to attack the subtrate even though the presence of the specific dehydrase could be demonstrated. The results are interpreted as indicating the existence of specific transport mechanisms for bringing the tartaric acids into the cell where the dehydrases can attack them. It appears that each isomer induces not only a specific dehydrase but a transport system as well (99).

The strain of *Pseudomonas* studied by Martin & Foster (100) exhibited a different adaptive behavior to the isomers of tartaric acid. Cells cultured on *meso*-tartrate oxidized *meso*-tartrate at once and all other isomers after a definite induction period. On the other hand, cells cultured on L-(+)- or D(−)-tartrate oxidized all isomers immediately. Sonic extracts displayed the same adaptive behavior as the intact cells from which they were obtained; therefore, permeability factors were excluded. The described be-

havior would be the result anticipated if the L- and the D-tartrates served as inducers for the synthesis of the *meso*-tartrate enzyme as well as the enzyme(s) which oxidizes the optically active isomers, but could not be utilized as substrate by the *meso*-tartrate enzyme (100).

The formation of cellulase in the mold, *Trichoderma viride*, was induced by cellulose and lactose, but not by a wide variety of other substrates (101). The inorganic ion composition of the medium had a pronounced effect on growth and enzyme production. Magnesium was necessary for good growth and cellulase production; but increased concentrations inhibited cellulase formation unless calcium was also present. Calcium stimulated enzyme production but had no effect on growth. Trace elements greatly stimulated the yield of enzyme; the best yields of cellulase were obtained in the presence of Fe, Mn, Zn, and Co. The function of the inorganic ions is not known; however, it is thought that a proper balance among them may be more important than their individual concentrations. The question of how an insoluble subtrate can induce the formation of an extracellular enzyme has not been answered. This phenomenon is not unique to cellulase. β-Glucuronidase induction by the thiolglucuronides is an example of adaptive enzyme formation without demonstrable incorporation of the inducer (102).

Ohmura & Hayaishi (103) presented evidence for the formation of different enzymes with the same biologic activity in response to two different inducers. An enzyme was isolated and purified from a strain of *Pseudomonas* grown on β-[imidazolyl-4(5)]acetic acid which was capable of hydrolyzing formylaspartic acid in the presence of $Fe^{+2}$ or $Co^{+2}$. A different enzyme was obtained from histidine-grown cells of *Pseudomonas* which also hydrolyzed formylaspartic acid in the presence of $Co^{+2}$ but was more active toward acyl derivatives of glutamic acid. The two enzymes also differed in physicochemical properties.

The bacterial decarboxylases so far studied are adaptive enzymes (104). The neutral amino acid decarboxylases of *Proteus vulgaris* studied by Ekladius *et al.* (105) are no exception. Valine, leucine, or isoleucine simultaneously induced enzymes capable of decarboxylating leucine, valine, norvaline, isoleucine, and α-amino-*n*-butyric acid. No additive effects were obtained by supplying two amino acids at the same time. From the induction behavior and other considerations the authors suggest that a single enzyme may be responsible for the decarboxylation of the five amino acids listed above. This situation differs distinctly from the demonstrable substrate specificity of the other known amino acid decarboxylases (104).

Neidhart & Magasanik have shown that glucose inhibits several inducible enzymes in *Aerobacter aerogenes* and were unsuccessful in reversing the inhibition by supplementing the minimal medium with amino acids, purines, pyrimidines, or vitamins (106). However, by placing the organism in a situation in which the synthesis of an inducible enzyme in the presence of glucose was a prerequisite for growth, they succeeded in reversing the

glucose effect (107). This was accomplished in two ways: (*a*) by substituting L-histidine for ammonium sulfate in the growth medium of a wild strain of *A. aerogenes,* and (*b*) by replacing L-glutamic acid with L-histidine in the glucose-containing medium of a glutamic acid-requiring mutant of *A. aerogenes.* In both cases, histidase production was necessary for growth. *A. aerogenes* cells growing on glucose and ammonium salts produce metabolic intermediates in excess of their biosynthetic needs (108). Under these conditions, L-histidine degradation is superfluous, and the enzymes responsible for its breakdown are not formed. All of the other enzymes, the formation of which is inhibited by glucose [tryptophanase, β-galactosidase, galactozymase, alanine deaminase, glutamic acid deaminase, serine deaminase, ornithine decarboxylase, aspartase, alcohol dehydrogenase, succinic dehydrogenase, formic dehydrogenase, *myo*-inositol dehydrogenase, glycerol dehydrogenase, cytochrome, and cytochrome oxidase (109)], function in a manner which furnishes the cell with metabolic building blocks and energy (107). Therefore, it is the products of glucose degradation which control the synthesis of enzymes which form these metabolites.

Yates & Pardee (110) showed that by varying conditions of culturing *E. coli,* the activities of three constitutive enzymes required for orotic acid synthesis could be varied markedly. The increased enzyme activity was the result of an actual increase in enzyme protein; in other words, the enzymes were made to behave like adaptive enzymes. In growing bacteria then, some system of control must be operative to impose on these enzymes the characteristics of constitutive enzymes. It was found that end products of their metabolic pathways inhibited enzyme formation. This method of control has been called "enzyme repression" and refers to control of enzyme synthesis which should be distinguished from control of enzyme activity (110). The control by arginine of the synthesis of the constitutive enzyme in *E. coli,* transcarbamylase (111), is another example of control by enzyme repression. A system of coordinated control of biosynthetic reactions must be operative under conditions of sustained exponential growth. The subject of control of enzyme activity has recently been reviewed by Pardee (112).

Yčas & Drabkin (113) have shown that in yeast cells exposed to oxygen there was an active biosynthesis of cytochrome-*c* and its hemin. The experiments demonstrate the adaptive character of the enzyme. The biosynthesis of another adaptive chromoprotein, catalase, was studied by Jensen (114) in a hemin-requiring, streptomycin-resistant mutant of *Micrococcus pyogenes* var. *aureus.* This strain of *Micrococcus* [like certain selected streptomycin-resistant mutants of *E. coli* (115)] synthesizes apocatalase in media free of hemin, and therefore provides a good system for studying the terminal synthesis of catalase. The data revealed that the combination of protein and prosthetic group is a complex reaction, requiring CoA and an unidentified substance derived from glucose. The implications are that a combining enzyme system is essential to this process (114).

*Lipides, sterols, and related compounds.*—The recent contribution of Hofmann *et al.* (116) concerns the effect of certain alterations in the composition of the culture medium on the fatty acid spectrum of *Lactobacillus delbrueckii*, and presents evidence for the biosynthetic pathway of lactobacillic acid. The addition of Tween-40 to the lipide-free growth medium containing biotin greatly stimulated fatty acid biosynthesis. The biotin content of the cells grown on media in which biotin had been replaced by oleic, *cis*-vaccenic, or lactobacillic acid was much less than control cells grown on media containing biotin. This finding supports previous conclusions that the fatty acids do not promote growth by serving as precursors for biotin. When biotin was replaced by oleic acid, the proportion of the ($C_{18} + C_{19}$) fraction was lower than in cells grown on the biotin or *cis*-vaccenic acid media; but this fraction was considerably more active in supporting growth of *L. delbrueckii* than pure lactobacillic acid. The implication is that oleic acid stimulates the biosynthesis of a saturated fatty acid which is more active than lactobacillic acid for *L. delbrueckii*. The absence of monounsaturated fatty acids in *L. delbrueckii* and *L. arabinosus* cells grown on lactobacillic acid and the increase in the $C_{19}$ fraction of cells grown on *cis*-vaccenic acid indicated that *cis*-vaccenic acid may be a precursor of lactobacillic acid. Portelance & Panisset (117) also found considerable variation in the lipides of *M. tuberculosis* with changes in cultural conditions.

Gaby *et al.* (118) studied the fate of unsaturated acids added to the culture medium of *Penicillium chrysogenum* and the lipide content of the harvested mycelium. Growth of the mold in the presence of oleic acid or some other long-chain unsaturated fatty acid resulted in an increase in the rate of oxidation of glucose and of saturated and unsaturated fatty acids. Sixty to 65 per cent of the long-chain unsaturated fatty acids were converted to linoleic acid by *P. chrysogenum* strains Q-176 and 47-133SL5, the remainder being oxidized. The phospholipides of these strains contain linoleic acid as the fatty acid portion. The cephalin fraction contains serine, ethanolamine, tyrosine, and an unidentified amino acid. The lecithin fraction yielded on hydrolysis seven ninhydrin-positive compounds in addition to choline.

Mevalonic acid has been shown to be a precursor of β-carotene in *Phycomyces blakesleeanus* by direct incorporation of the $C^{14}$-labeled lactone (119) and by experiments in which mevalonic acid was shown to be as efficient as leucine in diluting the incorporation of 2-$C^{14}$-acetate into β-carotene (120). It is also converted to squalene by a particle-free extract from baker's yeast (121). Thus, the function of mevalonic acid in microorganisms appears to be similar to that in animals (122), namely, that of a precursor of steroids and other substances derived in some way from isoprene units.

The effect of 3-$C^{14}$-leucine and 4-$C^{14}$-leucine on carotene formation in growing cultures of *Phycomyces blakesleeanus* has been studied by Yokoyama *et al.* (123). The stimulatory effect of leucine on carotene syn-

thesis was shown to be due to the incorporation of leucine fragments into the molecule. Beta-carotene biosynthesis has also been studied in *P. blakesleeanus* by Reichel & Wallis (124) and in *Penicillium sclerotiorum* by Mase *et al.* (125).

The formation of the sterol and lipide fractions in *Saccharomyces cerevisiae* was studied by Kodicek & Ashby (126). The sterol-starved yeast was incubated aerobically, in phosphate-acetate buffer with methyl labeled $C^{14}$-acetate; and aliquots of cells were removed and analyzed for sterols at intervals during a period of 24 hr. Radioactive carbon was found in ergosterol, zymosterol, lanostadienol, squalene, farnesol, two unidentified minor sterols, and three unidentified substances. The relationship of the concentration of these materials to the time of their formation and disappearance is consistent with the proposed pathway of sterol biosynthesis in yeast. The $C^{14}$-fatty acids increased proportionately with the increase in unsaponifiable matter, indicating that they were not being shunted back into the formation of steroids. However, the fatty acids of yeast were able to serve as carbon sources for isoprenoid units when added during the aerobic incubation period.

The source of the carbon atoms on the side chains of $C_{28}$, $C_{29}$, and $C_{31}$ steroids has received the recent attention of investigators in the field since these carbons are not supplied by squalene (127). Dauben *et al.* (127) showed by isotope experiments that formate can serve as the source of $C_{28}$ in ergosterol or the triterpene, eburicoic acid. Alexander *et al.* (128) showed that the methyl group of methionine was an even better source of $C_{28}$ than formate.

An interesting aspect of steroid biosynthesis is the use of microorganisms for the synthesis of isotopically labeled sterols (129) in good yield from labeled acetate and for readily achieving transformations of steroids (130 to 133) which would be difficult, if not impossible, by known chemical reactions. The fungi have been particularly useful for this purpose. The application of microbial transformations of steroids to the synthesis of hormones has recently been reviewed by Eppstein *et al.* (134).

*Biosynthesis of miscellaneous cell constituents and exudates.*—As a result of studies on the iron and porphyrin metabolism of *M. lysodeikticus*, Townsley & Neilands (135) found that, like other microorganisms studied, coproporphyrin rather than protoporphyrin was excreted when low levels of iron were present in the growth medium. Very low levels (0.18 μg. atoms/l. medium) of iron supported very little growth, and consequently, little porphyrin production; and high levels (8.85 μg. atoms/l. medium) inhibited porphyrin accumulation. The types and amounts of porphyrins formed by cell lysates depended upon the initial iron concentration of the growth medium. Spectral analysis of cell lysates during porphyrin synthesis from δ-aminolevulinic acid revealed that compounds with the properties of porphyrinogens were formed rather than porphyrins.

That the hematin compounds of aerobic bacteria are quite susceptible to cultural conditions is further demonstrated by the finding of Chaix &

Petit (136) that the absorption spectrum of hematin compounds of *Bacillus subtilis* was easily modified by varying the carbon source of the growth medium, and consequently, the rate of growth. The supply of oxygen during growth was constant and plentiful; and all spectral measurements were made during the exponential growth phase.

Probably the most extensively studied cell exudate from the point of view of biosynthesis is penicillin. The relevant literature up to 1957 has been reviewed by Ganapathi (137). The recent investigation of Arnstein & Clubb (138) has confirmed earlier work which indicated that valine is a precursor of the penicillamine moiety of penicillin by *Penicillium chrysogenum* and demonstrated that the carbon chain of valine was used intact for this purpose. β-Hydroxyvaline was not an intermediate in the synthesis of penicillin from valine. Demain (139) found that lysine markedly inhibited penicillin formation in *P. chrysogenum* and that the inhibition was not reversed by arginine.

The biosynthesis of cellulose by *Acetobacter* was studied by Greathouse (140). Delayed addition of glucose-1-$C^{14}$ to the growing culture of *A. xylinum* favored its polymerization to cellulose without prior cleavage. Hydrolysis of cellulose formed from glucose-6-$C^{14}$ yielded glucose with 82 per cent of the label in the original position 6. When glycerol-1, 3-$C^{14}$ was the sole carbon source, the label was unevenly distributed in all 6 positions. Hexose phosphates have been identified as intermediates in the biosynthesis of cellulose from D-glucose. The results led Greathouse to conclude that two major mechanisms appear to be involved: (*a*) direct polymerization, possibly involving phosphorylation, and (*b*) cleavage of the hexose and resynthesis of hexose phosphates from trioses such as glyceraldehyde.

Hauser & Karnovsky (141) studied the biosynthesis of the glycolipide formed by *Pseudomonas aeruginosa* when grown on a glycerol or fructose medium. The glycolipide is composed of two moles each of L-rhamnose and β-hydroxydecanoic acid. Studies on the incorporation of radioactivity from glycerol-α-$C^{14}$, glycerol-β-$C^{14}$, acetate-1-$C^{14}$, and $C^{14}O_2$ into rhamnolipide yielded data from which the following conclusions were drawn: (*a*) glycerol can furnish all of the carbon of rhamnolipide while acetate can supply only the β-hydroxydecanoic acid carbon; (*b*) the rhamnose portion of the rhamnolipide molecule seems to arise from the condensation of two 3-carbon units formed from glycerol without cleavage of its carbon-carbon bonds; (*c*) the carbon skeleton of hydroxydecanoic acid appears to be synthesized from 2-carbon units; (*d*) $CO_2$ does not furnish rhamnolipide carbon.

The suggestion of Leloir & Cardini (142), that uridine diphosphate-N-acetylglucosamine (UDPAG )might be involved in chitin biosynthesis, has been confirmed in the work of Glaser & Brown (143). They showed that radioactive UDPAG, labeled either in the glucosamine chain or in the acetyl group, when incubated with cell-free extracts from *Neurospora crassa* gave rise to radioactive chitin. This evidence adds another reaction to the lengthening list in which nucleotide-linked sugars have been shown to serve as glycosyl donors (143).

*Unidentified growth factors.*—The Hiochi bacteria, the agents responsible for the spoilage of Japanese rice wine, are classified at present as lactic acid bacteria. They are difficult to culture and maintain, and for this reason their nutrition is discussed under this heading. Among 64 strains tested by Kitahara *et al.* (144), only a few members grew in glucose-yeast extract media, but all strains grew in saké containing liver extract or in a semi-synthetic medium containing hiochic acid.[3] However, growth in liquid media was very slow. Teramoto *et al.* (147) reported that these organisms require folic acid, which is replaceable by thymine plus purine bases, and that growth is stimulated by a partial hydrolysate of rice protein, the active component of which they presume to be a peptide (148).

*Hemophilus (Bordetella) pertussis* is an unstable organism and degenerates in laboratory culture (149). In spite of extensive studies on its nutrition, reviewed by Rowatt (150), rapid and luxuriant growth of the organism cannot yet be obtained without adding blood to the medium (151).

Bailey (152) has studied the cultural characteristics of *Streptococcus pluton and Bacterium eurydice.* The former organism has no critical requirements for aerobic growth other than glucose, fructose, or sucrose for a carbon source. Anaerobically, however, it requires in addition to the carbohydrate source, a high inorganic phosphate concentration, a low molar ratio of sodium to potassium, and unidentified factors provided by yeast extract. The requirements of *B. eurydice* are similar.

Six members of the genus *Arthrobacter* have been studied by Burton (153) and compared to *Arthrobacter terregens,* the organism first reported (154) to require the "terregens factor." The requirement of all of the organisms for the "terregens factor" could be replaced, as it was for *A. terregens,* by iron-containing compounds such as hemin, coprogen, and ferrichrome; and also by the antibiotic, aspergillic acid. Although the terregens factor, ferrichrome, and coprogen have similar biologic activity for these organisms and others as well, they differ in some of their chemical and physical properties (155).

Evidence for the presence of a functionally active analogue of lipoic acid (6,8-dithiooctanoic acid) in the water mold, *Allomyces macrogynus,* was obtained by Machlis (156). Growth of the mold was inhibited by low concentrations of lipoic acid, also by 8-methyl-6, 8-dithiooctanoic acid; but 5,8-dithiooctanoic acid, glutathione, thioglycolate, cystine, and cysteine were inactive. The inhibition was prevented by acetate, pyruvate, aspartate, glutamate, arginine, and proline. Sodium arsenite inhibited growth and respiration but 2,3-dimercaptopropanol (BAL) failed to relieve this inhibition. The involvement of a form of lipoic acid is indicated by these data.[4]

[3] Hiochic acid was isolated by Tamura from the culture broth of *Aspergillus oryzae* and shown to be a growth factor for *Lactobacillus heterohiochii* (145). He has since identified the growth factor as β-hydroxy-β-methyl-δ-valerolactone and shown it to be identical to mevalonic acid (146).

[4] The chemistry and function of lipoic acid has recently been reviewed by Reed (157).

*Stimulatory factors.*—Some of the factors which stimulate growth of microorganisms under a defined set of conditions are familiar, well-known compounds; others have not yet been identified. For reasons discussed by Kitay & Snell (158), these effects are more readily observable when small inocula are used. Among the reports of the past year concerning growth stimulation is one by Sheffner & Adachi (159) showing that the addition of lipoic acid to the culture medium stimulated growth of *S. faecalis* in the presence of suboptimal concentrations of isoleucine, valine, and to a lesser extent arginine. Acetate did not enhance growth under these conditions. Because the recovery of leucine, isoleucine, and valine was improved by including lipoic acid in the medium, it was recommended that the assay medium for these amino acids be supplemented with lipoic acid.

A peptide was isolated from pancreas extract by McAnelly & Speck (160) which stimulated growth of *L. casei.* The active peptide was hydrolyzed and 13 amino acids identified; three ninhydrin-positive areas did not correspond to glutamine or asparagine and still remain unidentified.

The human tubercle bacillus has been difficult to cultivate in agar medium without the addition of egg yolk or whole egg or the aseptic addition of bovine serum or a fraction obtained from it (161). Yamane (161) has isolated the crystalline stimulatory factor from egg yolk; it is heat-stable and soluble in ether or chloroform. When added to a purified agar medium, it is required in concentrations above .01 per cent to support growth of *Mycobacterium tuberculosis* var. *hominis* from small inocula.

Since the initiation of nutritional studies on lactic acid bacteria, it has been observed that heat sterilization of the culture medium results in growth stimulation of the test organism. The effect has been traced to glucose, for when a sterile solution of this component is added aseptically to the remainder of the previously autoclaved medium, growth fails to occur or is much delayed. Numerous reports have appeared in the literature concerning the stimulatory effect on microorganisms of heating glucose or some other fermentable carbohydrate with components of the culture medium, salts (phosphate in particular), amino acids, organic acids, or alkalies; and many attempts have been made to identify the active products of this reaction. The heat degradation product(s) of glucose which have been identified as stimulatory factor(s) vary from one organism to another. The organisms which have been reported to be stimulated by autoclaving the growth medium and the substances which partially duplicate this effect are summarized in Table 1. The production of growth inhibitory substances under these conditions has also been reported (182 to 186).

The possible reactions which glucose can undergo when heated in the presence of components of a nutrient medium are so numerous that the recorded effects of these reactions on growth of microorganisms have probably not approached exhaustion. Recent studies (187, 188) on the nonenzymic browning reaction have yielded intermediates which should probably be tested in those instances where the heat degradation products have resisted identification.

TABLE I

STIMULATORY FACTORS PRODUCED BY HEAT STERILIZATION OF COMPONENTS OF THE CULTURE MEDIUM

| Organisms stimulated | Substances capable of simulating heat effects when added to unheated medium | References |
|---|---|---|
| *Saccharomyces cerevisiae* | unidentified | (162) |
| *Aerobacter pectinovorum*, *Serratia marcescens*, *Escherichia coli* | " | (163) |
| Streptococci and Lactobacilli | " | (164, 165) |
| Yeast | β-alanine | (166, 167) |
| *Streptococcus salivarius* | pyruvic acid, acetaldehyde | (168) |
| *Streptococcus faecalis* | ascorbic acid, cysteine, thioglycolate | (169) |
| *Lactobacillus bulgaricus* | pyruvate | (170) |
| *Lactobacillus leichmannii* | thioglycolate, asparagus extract | (171) |
| *Streptococcus faecalis*, *Lactobacillus arabinosus* | ascorbic acid | (172) |
| *Lactobacillus fermenti* | asparagus and other plant juices, fructose+maltose+reducing agents | (173) |
| *Lactobacillus gayoni* | N-D-glycosylglycine ethyl ester, high levels of yeast extract, ascorbic acid | (174) |
| *Lactobacillus fermenti*, *Leuconostoc mesenteroides*, *Streptococcus lactis*, *Bacillus subtilis*, *Micrococcus pyogenes*, *Aerobacillus polymyxa* | unidentified | (175) |
| *Streptococcus faecalis* | acetate* | (176) |
| *Propionibacteria* | N-D-glycosylglycine, high levels of yeast extract | (177) |
| *Bacillus globigii* | unidentified | (178) |
| *Bacillus globigii* | chelating agents | (179) |
| *Lactobacillus lactis* | formic acid | (180) |
| *Bacillus subtilus* (spore germination) | unidentified | (181) |

* Shockman (176) did not identify the stimulatory substance produced as acetate when glucose was autoclaved with the medium, however, Guirard & Snell (unpublished data) have shown that under similar conditions, employing *Lactobacillus*(30a) as assay organism, acetate is formed.

The importance of the polyamines (putrescine, spermine, spermidine) in microbial nutrition has been briefly reviewed by Kihara & Snell (189). How they function, however, is still not known. Kihara & Snell (189) observed that crude enzymatic digests of casein were more effective than digests of purified casein prepared with recrystallized trypsin in stimulating growth of *L. casei* from small inocula. Under the conditions of assay em-

ployed, addition of ascorbic, guanylic, and adenylic acids enhanced growth in the presence of either casein digest. The stimulatory activity of the nonpeptidic fraction of the crude enzymatic digest of casein arose mainly from its content of spermine and sperimidine. High concentrations of these polyamines inhibited growth of *L. casei.*

Previous isotopic experiments on the biosynthesis of the polyamines with growing cultures of *E. coli,* a mutant of *Aspergillus nidulans,* and *Neurospora crassa* indicated that putrescine (190) and methionine (191) were precursors of the 4- and 3-carbon chains, respectively, of spermine and spermidine. Recently Tabor *et al.* (192), using cell-free extracts of *E. coli,* have shown that 1,4-$C^{14}$-putrescine and 2-$C^{14}$-methionine, but not methyl or carboxyl-labeled methionine, were incorporated into spermidine through an intermediate thought to be adenosylmethionine. Greene (193) has published the details of his work on the incorporation of the carbon chain of methionine into the 3-carbon chain of spermidine in *N. crassa.*

## Growth Inhibition Studies

Recent publications have shown that inhibition studies on intact microbial cells or cell particles, although yielding many fruitful results, have also, by neglecting to consider cellular transport, led to many erroneous conclusions (1, 3, 75).

Studies on amino acid interrelationships have been particularly susceptible to misinterpretation. The paper by Hirsch (194) is relevant, in which the results of inhibition studies of homoserine-requiring mutants of *E. coli* by an analogue were analyzed by the inhibition analysis method of Shive (195). Attempts to confirm the validity of these conclusions by an independent method of study revealed their inaccuracy. The experimental results were these: pentaserine (α-amino-δ-hydroxyvaleric acid) did not inhibit wild type *E. coli* or threonine-requiring mutants, but did inhibit competitively growth of mutants requiring homoserine. However, in the presence of a mixture of threonine and methionine, growth of the mutants was not inhibited by pentaserine. An inhibition analysis of the data led to the conclusion that pentaserine inhibits the synthesis of threonine and methionine. Experiments were then undertaken to determine directly the effect of pentaserine on threonine synthesis by using cell suspensions and extracts. Pentaserine inhibited threonine synthesis by cell suspensions but not by cell extracts of the homoserine-requiring mutant. These results were explained on the basis of a competition between pentaserine and homoserine for entrance into the cell via the specific transport system; pentaserine inhibition is apparently completely unrelated to threonine synthesis.

As a result of studies on the microbiologic properties of a thiophene analogue of carnosine, β-alanyl-β-2-thienylalanine, Dunn (196) found that it inhibited growth of *E. coli* 9723. The inhibition was reversed noncompetitively by phenylalanine and glycylphenylalanine, competitively by carnosine and β-alanylphenylalanine, and not at all by histidine, β-alanine, or an equimolar mixture of histidine and β-alanine. The β-alanyl peptides

were not hydrolyzed by resting cell preparations of *E. coli* 9723. The results were interpreted as indicating that the peptides were utilized as such without previous conversion to constituent amino acids. The data appear to be consistent with the suggestion that β-alanyl-β-2-thienylalanine exerts its influence as a peptide inside of the cell; however, carnosine and β-alanylphenylalanine may relieve the inhibition by preventing the structurally related analogue from entering the cell.

Aposhian *et al.* (197) studied the inhibitory effect of penicillamine on acid production by *Leuconostoc mesenteroides* P-60. The reversal of inhibition by serine led the authors to conclude that penicillamine was competing with serine or a closely related metabolic product. The effect of penicillamine on the activity of vitamin $B_6$ should not be overlooked in these experiments. The antivitamin $B_6$ action of L-penicillamine in animals has been reported (198).

The feed-back mechanism of control of biosynthetic processes as it applied to the synthesis of adaptive and some constitutive enzymes has been discussed. The feed-back mechanism as applied to control of the synthesis of a precursor by its end products has been studied by Gots (199). Two purine-requiring strains of E. coli B were used, B-96 and B-96/1t; the latter strain had an additional requirement for tryptophan. The synthesis of AICA (5-amino-4-imidazolecarboxamide) was followed in growing cultures with strain B-96 and in nonproliferating systems with strain B-96/1t. In growth experiments, maximum accumulation of AICA occurred at concentrations of the purine or derivative which permitted 30 to 50 per cent of the maximal growth; concentrations of the growth factors which produced maximal growth suppressed AICA accumulation. In nonproliferating cell suspensions actively synthesizing the aminoimidazole, the addition of any purine or its derivative which could satisfy the growth requirement under cultural conditions (and only those) significantly or completely inhibited the formation of AICA. The degree of inhibition was proportional to the log of the concentration of purine.

The effects of 6-mercaptopurine on biosynthesis in *E. coli* was studied by Bolton & Mandel (200) and in *L. casei* by Balis *et al.* (201). Bolton & Mandel found that the utilization of acetate for protein and lipide synthesis was strongly suppressed and that nucleic acid synthesis was reduced to 50 per cent of that of control cultures. The authors suggested that 6-mercaptopurine exerted its effect by interfering with the function and synthesis of purine-containing cofactors. Balis *et al.* found that in addition to inhibiting growth of *L. casei*, the drug produced an alteration in the pattern of utilization of the purine bases.

The toxic action of fatty acids and detergents has most frequently been ascribed to physicochemical effects (202). Camien & Dunn (202), however, found that the inhibition of growth of lactic acid bacteria produced by saturated fatty acids containing 12 to 20 carbon atoms was readily reversed by lactobacillic acid and several unsaturated fatty acids. The results suggested antimetabolite-metabolite relationships rather than purely physico-

chemical phenomena. Minami (203), on the other hand, found that a few seconds' exposure of tubercle bacilli to $10^{-3}$ *M* oleate was sufficient to produce a strong adsorption of oleate on the bacteria with the attendant observed effects of reduction in the number of viable cells, and inhibition of oxygen uptake and dehydrogenase activity. The inhibitory effects were not observed when the oleate-treated cells were disrupted by grinding. The results suggested that the surface structure of the cells was concerned in the inhibitory effect of oleate.

Reports have appeared in the recent literature, concerning the inhibition of growth of strains of *M. tuberculosis* by high carbon dioxide tension (204); the effect of pentamidine on growth of *E. coli* (205); the preparation and bacteriostatic activity of substituted ureas (206); the action of thymidine analogues on the growth of *E. coli* (207); the inhibitory effect of vitamin $B_6$ derivatives structurally related to thiamine (208); inhibition of growth, respiration, phosphate utilization, and nitrogen fixation in *A. vinelandii* (209, 210, 211).

## Miscellaneous

*Sporulation, germination, and outgrowth.*—Requirements for germination of spores are distinct from those of growth of aerobic spore-forming bacteria and, in some cases, are more exacting (212). The experiments of O'Brien & Campbell (213) on germination and outgrowth of spores of several strains of bacilli support the above generalization.

Germination and postgerminative development (outgrowth) of *Bacillus megaterium* spores was studied by Hyatt & Levinson (214). The nutritional requirements changed qualitatively as the germinated spore swelled, emerged elongated, and divided. In addition to glucose and a nitrogen source, $Mn^{+2}$ was found to be necessary for rapid spore germination and $SO_4^{-2}$ for development subsequent to germination. Sulfate was replaceable by other inorganic and organic sources of sulfur. Germination of the spores was not affected by $Co^{+2}$ and $Ni^{+2}$ but postgerminative development was inhibited.

The effect of metal ions and anions on germination and outgrowth of *B. cereus* spores (215) and on germination of fungus spores (216) has been investigated.

The stimulation of germination of wheat rust spores by the volatile aldehyde, pelargonaldehyde, was reported by French & Weintraub (217). The aldehyde is thought to arise in the spores from oxidation of fat.

The nutrition and sporulation of the pathogenic ascomycete, *Allescheria boydii* (218) and the requirements for production and germination of spores of anaerobic bacteria (219) have been studied.

*Nutrition and morphology.*—The presence of long or abnormal forms of bacteria in a culture are an indication of the inadequacy or the toxicity of the growth medium (220). Nutritionally, they may be induced by starvation (nitrogen, magnesium, etc.) or by the addition of such substances as lithium chloride, amino acids, or antibiotics (220). These inducing agents appear to be of a type which affect either cell division or the cell wall.

The recent publication of Spoerl *et al.* (221) describes the effect of zinc and amino acids in producing long, clumped cells of *Ustilago sphaerogena.* They attribute the effect to one on cell division.

An interesting effect of vitamin $B_6$ deficiency in *L. arabinosus* cells was observed by Holden & Holman (222). Serial passage of the cells in a vitamin $B_6$-deficient medium resulted in progressively less growth and the development of a population of cells of abnormal shape. The abnormality was completely reversible by one passage in the medium supplemented with pyridoxamine. The vitamin $B_6$-deficient cells were more rapidly disintegrated by sonic vibration and showed larger changes in optical density in solutions of varying tonicity than the vitamin $B_6$-sufficient cells. The observations suggested to the authors that the morphologic changes may be a consequence of an alteration in the composition or structure of the cell wall.

The role of diaminopimelic acid in maintaining the integrity of the cell, presumably by serving as a structural component of the cell wall, has been reported (223, 224). A deficiency of this amino acid results in the production of protoplasts or complete disintegration of the cell. The biochemistry of the bacterial cell wall has recently been reviewed (225).

Sall *et al.* (226) found that germination of spores of *B. megaterium* on a nitrogen-deficient medium resulted in the production of cells which differed chemically and morphologically from those germinated on a medium adequate in nitrogen. The resulting vegetative cells were deficient in total nitrogen and RNA but higher in lipide content. They were enlarged and relatively transparent.

*Microbiologic assays.*—It is well recognized that a thorough knowledge of the nutrition of the organism employed for assay is of prime importance to the validity of the assay. The advantages and disadvantages of, and the factors affecting microbiologic assays, employing turbidimetric methods have recently been reviewed by Gavin (227).

An improved medium for the assay of thiamine with *Lactobacillus fermenti* (228) has been reported. Deibel *et al.* (229) developed a method for thiamine using *Lactobacillus viridescens* which they claim is more specific, more convenient, and less subject to interfering substances than currently available methods.

Camien *et al.* (230) have published a method of determining L- and D-glutamic acids by a combined microbiologic and racemization procedure which is an improvement over their previous method.

A method is described by Wolff *et al.* (231) by which pantothenic acid plus pantetheine may be determined. Under these conditions pantothenic acid and pantetheine were equally active on the molar basis for *L. arabinosus.* Bound pantothenic acid was released from CoA by incubation of the aqueous tissue extract with phosphatase from calf intestinal mucosa.

Seidler *et al.* (232) have re-evaluated the nutritional requirements of *L. acidophilus* R-26 after finding that the growth response of the organism was much improved when yeast or liver extract was added to the medium

described by Hoff-Jørgensen (233). Based on the results of these studies, a modified assay medium was formulated which improved the assay for deoxyribonucleic acid. Sneider & Potter (234) showed that *L. acidophilus* R-26 was unable to utilize pyrimidine deoxynucleoside polyphosphates. However, they suggested that it should be possible to convert these compounds enzymatically to deoxynucleosides or deoxynucleotides to make them available to the organism.

A microbiologic method for the screening of compounds of potential interest in the chemotherapy of leukemia was described by Guthrie *et al.* (235). Another study initiated for reasons of a similar nature was that of Adelson *et al.* (236) in which certain lipides and lipide derivatives were tested as growth substrates and for their effects on oxygen uptake by an actively myelinolytic actinomycete. Substances with antidemyelinating properties *in vitro* could then be tested clinically for their effects against multiple sclerosis and other demyelinating diseases. The study was more informative regarding the metabolism of actinomycetes than in revealing antidemyelinating agents effective in the test system.

The available microbiologic methods for determining trace metals are summarized and evaluated in the excellent review by Snell (237). The requirements of microorganisms for inorganic ions and their function in nutrition is also discussed.

Investigations of the inhibitory effects of antibiotics on microbial growth are far too extensive to be included in this review. Especially interesting in this regard are the papers concerning the mode of action of antibiotics, penicillin in particular. Another topic which must be omitted is the use of elective (enrichment) culture technique to obtain microorganisms capable of degrading complex molecules (sterols, bile acids, polynuclear hydrocarbons) and also less complex compounds (nicotinic acid) for the purpose of studying degradative pathways. The maintenance of virulence in cultures of pathogenic bacteria carried in the laboratory is largely a nutritional problem; however because of space limitations and the probability that this subject will be covered in another review in this volume, it will not be discussed here. For similar reasons, purely metabolic studies have been omitted. Finally, it is also regrettable that space will not permit inclusion of papers concerned with the effect of nutritional factors on enzyme activation or pigmentation of microbial cells.

## LITERATURE CITED

1. Cohen, G. N., and Monod, J., *Bacteriol. Rev.*, **21**, 169–94 (1957)
2. Mitchell, P., *Nature*, **180**, 134–36 (1957)
3. Davis, B. D., *Enzymes: Units of Biological Structure and Function*, 509–22 (Academic Press, Inc., New York, N.Y., 624 pp., 1956)
4. Rickenberg, H. V., *Biochim. et Biophys. Acta*, **25**, 206–7 (1957)
5. Englehard, W. E., Uyeno, S., and Pivnick, H., *J. Bacteriol.*, **73**, 206–9 (1957)
6. Rowlands, D. A., Gale, E. F., Folkes, J. P., and Marrian, D. H., *Biochem. J.*, **65**, 519–26 (1957)
7. Gale, E. F., and Folkes, J. P., *Biochem J.*, **67**, 507–17 (1957)

8. Kornberg, H. L., and Krebs, H. A., *Nature*, **179**, 988–91 (1957)
9. Campbell, J. J. R., Smith, R. A., and Eagles, B. A., *Biochim. et Biophys. Acta*, **11**, 594 (1953)
10. Smith, R. A., and Gunsalus, I. C., *J. Am. Chem. Soc.*, **76**, 5002–3 (1954)
11. Saz, H. J., and Hillary, E. P., *Biochem. J.*, **62**, 563–69 (1956)
12. Wong, D. T. O., and Ajl, S. J., *J. Am. Chem. Soc.*, **78**, 3230–31 (1956)
13. Kornberg, H. L., and Madsen, N. B., *Biochim. et Biophys. Acta*, **24**, 651–53 (1957)
14. Alexander, J. K., *J. Bacteriol.*, **74**, 168–70 (1957)
15. Lloyd, R. P., *J. Gen. Microbiol.*, **16**, 2P (1957)
16. Van Uden, N., and Assis-Lopes, L., *Portugaliae Acta Biol., Sér. A*, **4**, 323–27 (1957); *Chem. Abstr.*, **51**, 18092 (1957)
17. Smith, P. F., *J. Bacteriol.*, **73**, 91–95 (1957)
18. Smith, P. F., *J. Bacteriol.*, **74**, 801–6 (1957)
19. Fuchs, A. R., and Bonde, G. J., *J. Gen. Microbiol.*, **16**, 317–29 (1957)
20. Wolin, H. L., and Naylor, H. B., *J. Bacteriol.*, **74**, 163–67 (1957)
21. Pereira, J. N., and Morgan, M. E., *J. Bacteriol.*, **74**, 710–13 (1957)
22. Golueke, C. G., *J. Bacteriol.*, **74**, 337–43 (1957)
23. Taber, W. A., and Vening, L. C., *Can. J. Microbiol.*, **3**, 1–12 (1957)
24. Mergenhagen, S. E., and Scherp, H. W., *J. Bacteriol.*, **74**, 749–56 (1957 )
25. McNall, E. G., and Atkinson, D. E., *J. Bacteriol.*, **74**, 60–66 (1957)
26. Husain, I., and McDonald, I. J., *Can. J. Microbiol.*, **3**, 745–51 (1957)
27. Kizer, D. E., Speck, M. L., and Aurand, L. W., *J. Bacteriol.*, **69**, 16–19 (1955)
28. Simmonds, S., and Miller, D. A., *J. Bacteriol.*, **74**, 775–83 (1957)
29. Jebb, W. H. H., and Tomlinson, A. H., *J. Gen. Microbiol.*, **17**, 59–63 (1957)
30. Bulmash, J., and Weaver, R. H., *J. Bacteriol.*, **74**, 110 (1957)
31. Humphreys, T. W., and Costilow, R. N., *Can. J. Microbiol.*, **3**, 533–41 (1957)
32. Sobotka, H., Baker, H., Luisada-Oppen, A. V., and Hutner, S. H., *Rev. fermentations et inds. aliment.*, **12**, 51–56 (1957); *Chem. Abstr.*, **51**, 14882 (1957)
33. Turner, E. M., *J. Gen. Microbiol.*, **16**, 531–33 (1957)
34. Tomlinson, N., and MacLeod, R. A., *Arch. Biochem. Biophys.*, **70**, 477–90 (1957)
35. Koser, S. A., and Thomas, J. L., *J. Bacteriol.*, **73**, 477–83 (1957)
36. Yanofsky, C., *J. Biol. Chem.*, **224**, 783–91 (1957)
37. Parks, L. W., and Douglas, H. C., *Biochim. et Biophys. Acta*, **23**, 207–8 (1957)
38. Ames, B. N., and Mitchell, H. K., *J. Biol. Chem.*, **212**, 687–96 (1955)
39. Hakala, M. T., and Welch, A. D., *J. Bacteriol.*, **73**, 35–41 (1957)
40. Nichol, C. A., *J. Biol. Chem.*, **207**, 725–32 (1954)
41. Seaman, G. R., and Sanders, F., *Science*, **125**, 398–99 (1957)
42. Ford, J. E., Gregory, M. E., and Holdsworth, E. S., *Biochem. J.*, **61**, 23P (1955)
43. Barchielli, G., Boretti, G., Julita, P., Migliacco, A., and Minghetti, A., *Biochim. et Biophys. Acta*, **25**, 452 (1957)
44. Ford, J. E., Holdsworth, E. S., and Kon, S. K., *Biochem. J.*, **59**, 86–93 (1955)
45. Ford, J. E., and Hutner, S. H., *Can. J. Microbiol.*, **3**, 319–27 (1957)
46. Goldberg, M. K., Hutner, S. H., and Ford, J. E., *Can. J. Microbiol.*, **3**, 329–34 (1957)
47. Krasna, A. I., Rosenblum, C., and Sprinson, D. B., *J. Biol. Chem.*, **225**, 745–50 (1957)
48. Southcott, B. A., and Tarr, H. L. A., *Can. J. Microbiol.*, **3**, 195–202 (1957)

49. Brown, G. M., and Snell, E. E., *J. Am. Chem. Soc.*, **75**, 2782–83 (1953)
50 Hoagland, M. B., and Novelli, G. D., *J. Biol. Chem.*, **207**, 767–73 (1954)
51. Brown, G. M., *J. Biol. Chem.*, **226**, 651–61 (1957)
52. Goodwin, T. W., and McEvoy, D., *Biochem. J.*, **67**, 16P (1957)
53. Tanner, F. W., Jr., Vojnovich, C., and Van Lanen, J. M., *Science*, **101**, 180–81 (1945)
54. Pope, S., Tomarelli, R. M., and György, P., *Arch. Biochem. Biophys.*, 362–66 (1957)
55. Yarbrough, H. F., Jr., and Clark, F. M., *J. Bacteriol.*, **73**, 318–23 (1957)
56. Charalampous, F. C., *J. Biol. Chem.*, **225**, 595–605 (1957)
57. Martin, J. K., and Batt, R. D., *J. Bacteriol.*, **74**, 225–30 (1957)
58. Markov, K. I., and Saev, G. K., *Zentr. Bakteriol. Parasitenk.*, **168**, 302–12 (1957)
59. Norris, J. R., and Jensen, H. L., *Nature*, **180**, 1493–94 (1957)
60. Keeler, R. F., *J. Bacteriol.*, **73**, 582–83 (1957)
61. Fuchs, A. R., and Bonde, G. J., *J. Gen. Microbiol.*, **16**, 330–40 (1957)
62. Kucera, S., and Wolfe, R. S., *J. Bacteriol.*, **74**, 344–49 (1957)
63. MacLeod, R. A., and Onofrey, E., *Can. J. Microbiol.*, **3**, 753–59 (1957)
64. Slotnick, I. J., *J. Bacteriol.*, **72**, 276–77 (1956)
65. Slotnick, I. J., and Weinfeld, H., *J. Bacteriol.*, **74**, 122–25 (1957)
66. Fink, R. M., Fink, K., and Henderson, R. B., *J. Biol. Chem.*, **201**, 349–55 (1953)
67. Cohen, S. S., and Barner, H. D., *J. Biol. Chem.*, **226**, 631–42 (1957)
68. Green, M., and Cohen. S. S., *J. Biol. Chem.*, **225**, 387–96 (1957)
69. Cohen, S. S., Lichtenstein, J., Barner, H. D., and Green, M., *J. Biol. Chem.*, **228**, 611–19 (1957)
70. Green, M., Barner, H. D., and Cohen, S. S., *J. Biol. Chem.*, **228**, 621–31 (1957)
71. Herrmann, R. L., and Fairley, J. L., *J. Biol. Chem.*, **227**, 1109–16 (1957)
72. Abelson, P. H., and Vogel, H. J., *J. Biol. Chem.*, **213**, 355–64 (1955)
73. Wright, L. D., Driscoll, C. A., Miller, C. S., and Skeggs, H. R., *Proc. Soc. Exptl. Biol. Med.*, **84**, 716–19 (1953)
74. Crawford, I., Kornberg, A., and Simms, E. S., *J. Biol. Chem.*, **226**, 1093–1101 (1957)
75. Magasanik, B., *Ann. Rev. Microbiol.*, **11**, 221–52 (1957)
76. Aaronson, S., and Rodriguez, E., *J. Bacteriol.*, **74**, 807–10 (1957)
77. Cowie, D. B., and Bolton, E. T., *Biochim. et Biophys. Acta*, **25**, 292–98 (1957)
78. Cowie, D. B., and Walton, B. P., *Biochim. et Biophys. Acta*, **21**, 211–26 (1956)
79. Smith, J. D., and Matthews, R. E. F., *Biochem. J.*, **66**, 323–33 (1957)
80. Mandel, H. G., and Markham, R., *Biochem. J.*, **65**, 25P (1957 )
81. Škoda, J., Hess, V. F., and Šorm, F., *Experientia*, **13**, 150–51 (1957)
82. Dunn, D. B., and Smith, J. D., *Biochem. J.*, **67**, 494–506 (1957)
83. Prusoff, W. H., *J. Biol. Chem.*, **226**, 901–10 (1957)
84. Mandel, H. G., *J. Biol. Chem.*, **225**, 137–50 (1957)
85. Gale, E. F., and McQuillen, K., *Ann. Rev. Microbiol.*, **11**, 283–316 (1957)
86. Brawerman, G., and Yčas, M., *Arch. Biochem. Biophys.*, **68**, 112–17 (1957)
87. Cowie, D. B., and Cohen, G. N., *Biochim. et Biophys. Acta*, **26**, 252–61 (1957)
88. Tuve, T. W., and Williams, H. H., *J. Am. Chem. Soc.*, **79**, 5830–31 (1957)
89. Mudd, S. H., and Cantoni, G. L., *Nature*, **180**, 1052 (1957)
90. Hartman, R. E., Zimmerman, L. N., and Rabin, R., *Can. J. Microbiol.*, **3**, 553–58 (1957)
91. Kornberg, H. L., *Biochim. et Biophys. Acta*, **25**, 200–2 (1957)

92. Yčas, M., and Brawerman, G., *Arch. Biochem. Biophys.*, **68**, 118–29 (1957)
93. Ben-Ishai, R., *Biochim. et Biophys. Acta*, **26**, 477–83 (1957)
94. Hahn, F. E., Schaechter, M., Ceglowski, W. S., Hopps, H. E., and Ciak, J., *Biochim. et Biophys. Acta*, **26**, 469–76 (1957)
95. Aronson, A. I., and Spiegelman, S., *Bacteriol. Proc. (Soc. Am. Bacteriologists)*, **57**, 138 (1957)
96. Barner, H. D., and Cohen, S. S., *J. Bacteriol.*, **74**, 350–55 (1957)
97. Pardee, A. B., *J. Bacteriol.*, **73**, 376–85 (1957)
98. Rickenberg, H. V., Cohen, G. N., Buttin, G., and Monod, J., *Ann. inst. Pasteur*, **91**, 829–57 (1956)
99. Shilo, M., and Stanier, R. Y., *J. Gen. Microbiol.*, **16**, 482–90 (1957)
100. Martin, W. R., and Foster, J. W., *J. Bacteriol.*, **73**, 683–84 (1957)
101. Mandels, M., and Reese, E. T., *J. Bacteriol.*, **73**, 269–78 (1957)
102. Stoeber, F., *Compt. rend.*, **244**, 950–52 (1957)
103. Ohmura, E., and Hayaishi, O., *J. Biol. Chem.*, **227**, 181–90 (1957)
104. Gale, E. F., *Advances in Enzymol.*, **6**, 1–32 (1946)
105. Ekladius, L., King, H. K., and Sutton, C. R., *J. Gen. Microbiol.*, **17**, 602–19 (1957)
106. Neidhardt, F. C., and Magasanik, B., *Biochim. et Biophys. Acta*, **21**, 324–34 (1956)
107. Neidhardt, F. C., and Magasanik, B., *J. Bacteriol.*, **73**, 253–59 (1957)
108. Neidhardt, F. C., and Magasanik, B., *Nature*, **180**, 801–2 (1956)
109. Strittmatter, C. F., *J. Gen. Microbiol.*, **16**, 169–83 (1957)
110. Yates, R. A., and Pardee, A. B., *J. Biol. Chem.*, **227**, 677–92 (1957)
111. Gorini, L., and Maas, W. K., *Biochim. et Biophys. Acta*, **25**, 208–9 (1957)
112. Pardee, A. B., in *The Enzymes*, 2nd ed. (Academic Press, Inc., New York, N.Y., in press)
113. Yčas, M., and Drabkin, D. L., *J. Biol. Chem.*, **224**, 921–33 (1957)
114. Jensen, J., *J. Bacteriol.*, **73**, 324–33 (1957)
115. Beljanski, M., and Beljanski, M., *Ann. inst. Pasteur*, **92**, 396–412 (1957)
116. Hofmann, K., Henis, D. B., and Panos, C., *J. Biol. Chem.*, **228**, 349–55 (1957)
117. Portelance, V., and Panisset, M., *Rev. can. biol.*, **16**, 112–23 (1957); *Chem. Abstr.*, **51**, 3739 (1957)
118. Gaby, W. L., Hadley, C., Kaminski, Z. C., *J. Biol. Chem.*, **227**, 853–61 (1957)
119. Braithwaite, G. D., and Goodwin, T. W., *Biochem. J.*, **67**, 14P (1957)
120. Braithwaite, G. D., and Goodwin, T. W., *Biochem. J.*, **66**, 31P (1957)
121. Amdur, B. H., Rilling, H., and Block, K., *J. Am. Chem. Soc.*, **79**, 2646–47 (1957)
112. Tavormina, P. A., Gibbs, M. H., and Huff, J. W., *J. Am. Chem. Soc.*, **78**, 4498–99 (1956)
123. Yokoyama, H., Chichester, C. O., Nakayama, T., Lukton, A., and Mackinney, G., *J. Am. Chem. Soc.*, **79**, 2029–30 (1957)
124. Reichel, L., and Wallis, M., *Naturwissenschaften*, **44**, 234 (1957)
125. Mase, Y., Rabourn, W. J., and Quakenbush, F. W., *Arch. Biochem. Biophys.*, **68**, 150–56 (1957)
126. Kodicek, E., and Ashby, D. R., *Biochem. J.*, **66**, 35P (1957)
127. Dauben, W. G., Fonken, G. J., Boswell, G. A., *J. Am. Chem. Soc.*, **79**, 1000–1 (1957)
128. Alexander, G. J., Gold, A. M., and Schwenk, E., *J. Am. Chem. Soc.*, **79**, 2967 (1957)

129. Glover, J., and Leat, W. M. F., *Biochem. J.*, **66**, 209–14 (1957)
130. Herzog, H. L., Gentles, M. J., Hershberg, E. B., Carvajal, F., Sutter, D., Charney, W., and Schaffner, C. P., *J. Am. Chem. Soc.*, **79**, 3921–22 (1957)
131. Greenspan, G., Schaffner, C. P., Charney, W., Herzog, H. L., and Hershberg, E. B., *J. Am. Chem. Soc.*, **79**, 3922–23 (1957)
132. Dodson, R. M., Goldkamp, A. H., and Muir, R. D., *J. Am. Chem. Soc.*, **79**, 3921 (1957)
133. Thoma, R. W., Fried, J., Bonanno, S., and Grabowich, P., *J. Am. Chem. Soc.*, **79**, 4818 (1957)
134. Eppstein, S. H., Meister, P. D., Murray, H. C., and Peterson, D. H., *Vitamins and Hormones*, **14**, 359–432 (1956)
135. Townsley, P. M., and Neilands, J. B., *J. Biol. Chem.*, **224**, 695–705 (1957)
136. Chaix, P., and Petit, J. F., *Biochim. et Biophys. Acta*, **25**, 481–86 (1957)
137. Ganapathi, K., *Experientia*, **13**, 172–75 (1957)
138. Arnstein, H. R. V., and Clubb, M. E., *Biochem. J.*, **65**, 618–27 (1957)
139. Demain, A. L., *Arch. Biochem. Biophys.*, **67**, 244–46 (1957)
140. Greathouse, G. A., *J. Am. Chem. Soc.*, **79**, 4505–7 (1957)
141. Hauser, G., and Karnovsky, M. L., *J. Biol. Chem.*, **224**, 91–105 (1957)
142. Leloir, L. F., and Cardini, C. E., *Biochim. et Biophys. Acta*, **12**, 15 (1953)
143. Glaser, L., and Brown, D. H., *J. Biol. Chem.*, **228**, 729–42 (1957)
144. Kitahara, K., Kaneko, T., and Goto, O., *J. Gen. Appl. Microbiol. (Tokyo)*, **3**, 102–10 (1957)
145. Tamura, G., *J. Gen. Appl. Microbiol. (Tokyo)*, **2**, 431–34 (1956)
146. Tamura, G., *Bull. Agr. Chem. Soc. Japan*, **21**, 202–3 (1957)
147. Teramoto, S., Hashida, W., and Endo, Y., *Hakkô Kôgaku Zasshi*, **35**, 242–46 (1957); *Chem. Abstr.*, **51**, 16687 (1957)
148. Teramoto, S., Hashida, W., and Endo, Y., *Hakkô Kôgaku Zasshi*, **35**, 32–37 (1957); *Chem. Abstr.*, **51**, 18100 (1957)
149. Jebb, W. H. H., and Tomlinson, A. H., *J. Gen. Microbiol.*, **17**, 59–63 (1957)
150. Rowatt, E., *J. Gen. Microbiol.*, **17**, 297–326 (1957)
151. Rowatt, E., *J. Gen. Microbiol.*, **17**, 279–96 (1957)
152. Bailey, L., *J. Gen. Microbiol.*, **17**, 39–48 (1957)
153. Burton, M. O., *Can. J. Microbiol.*, **3**, 107–12 (1957)
154. Lockhead, A. G., and Burton, M. O., *Can. J. Botany*, **31**, 7–22 (1953)
155. Neilands, J. B., *Bacteriol. Rev.*, **21**, 101–11 (1957)
156. Machlis, L., *Arch. Biochem. Biophys.*, **70**, 413–18 (1957)
157. Reed, L. J., *Advances in Enzymol.*, **18**, 319–47 (1957)
158. Kitay, E., and Snell, E. E., *Proc. Soc. Exptl. Biol. Med.*, **68**, 648–50 (1948)
159. Sheffner, A. I., and Adachi, R., *Arch. Biochem. Biophys.*, **72**, 163–68 (1957)
160. McAnelly, J. K., and Speck, M. L., *J. Bacteriol.*, **73**, 676–81 (1957)
161. Yamane, I., *J. Bacteriol.*, **73**, 334–37 (1957)
162. Fulmer, E. I., and Heussleman, B., *Iowa State Coll. J. Sci.*, **1**, 411–17 (1927)
163. Fulmer, E. I., Williams, A. L., and Werkman, C. H., *J. Bacteriol.*, **21**, 299–303 (1931)
164. Orla-Jensen, S., *Congr. intern. Laiterie., Compt. rend., IX Congr.* (Copenhagen, Denmark, July, 1931)
165. Orla-Jensen, A. D., *J. Soc. Chem. Ind. (London)*, **52**, 374–79 (1933)
166. Neilsen, N., and Dragys, J., *Compt. rend. trav. lab. Carlsberg, Sér. physiol.*, **22**, 447–80 (1940)

167. Neilsen, N., and Eistrup, K., *Compt. rend. trav. lab. Carlsberg, Sér. physiol.*, **23**, 79–92 (1940)
168. Smiley, K. L., Niven, C. F., Jr., and Sherman, J. M., *Bacteriol.*, **45**, 445-54 (1943)
169. Rabinowitz, J. C., and Snell, E. E., *J. Biol. Chem.*, **169**, 631–42 (1947)
170. Snell, E. E., Kitay, E., and Hoff-Jørgensen, E., *Arch. Biochem.*, **18**, 495–510 (1948)
171. Hoffmann, C. E., Stokstad, E. L. R., Hutchings, B. L., Dornbush, A. C., and Jukes, T. H., *J. Biol. Chem.*, **181**, 635–44 (1949)
172. Rose, D. and Peterson, R., *Can. J. Research*, [B]**27**, 428–36 (1949)
173. Snell, N., and Lewis, J. C., *J. Bacteriol.*, **65**, 671–77 (1953)
174. Rogers, D., King, T. E., and Cheldelin, V. H., *Proc. Soc. Exptl. Biol. Med.*, **82**, 140–44 (1953)
175. Ramsey, H. H., and Lankford, C. E., *J. Bacteriol.*, **72**, 511–18 (1956)
176. Shockman, G. D., *J. Bacteriol.*, **72**, 101–4 (1956)
177. Field, M. F., and Lichstein, H. C., *J. Bacteriol.*, **73**, 96–99 (1957)
178. Sergeant, T. P., Lankford, C. E., Traxler, R. W., *J. Bacteriol.*, **74**, 728–36 (1957)
179. Lankford, C. E., Kustoff, T. Y., and Sergeant, T. P., *J. Bacteriol.*, **74**, 728–48 (1957)
180. Auclair, J. E., and Portmann, A., *Nature*, **179**, 782 (1957)
181. Hachisuka, Y., Asano, N., and Kato, N., *Nippon Saikingaku Zasshi*, **10**, 33–35 (1955); *Chem. Abstr.*, **51**, 12211 (1957)
182. Lewis, I. M., *J. Bacteriol.*, **19**, 423-33 (1930)
183. Schuhardt, V. T., Rode, L, J., Oglesby, G., and Lankford, C. E., *J. Bacteriol.*, **63**, 123-28 (1952)
184. Lankford, C. E., Ravel, J. M., and Ramsey, H. H., *Appl. Microbiol.*, **5**, 65–69 (1957)
185. Finkelstein, R. A., and Lankford, C. E., *Appl. Microbiol.*, **5**, 74–79 (1957)
186. Viswanathan, L., and Sarma, P. S., *Nature*, **180**, 1370–71 (1957)
187. Kato, H., *Bull. Agr. Chem. Soc. Japan*, **20**, 273–78 (1956)
188. Richards, E. L., *Biochem. J.*, **64**, 639-44 (1956)
189. Kihara, H., and Snell, E. E., *Proc. Natl. Acad. Sci. U. S.*, **43**, 867–71 (1957)
190. Tabor, H., Rosenthal, S. M., and Tabor, C. W., *Federation Proc.*, **15**, 367 (1956)
191. Greene, R. C., *Federation Proc.*, **16**, 189 (1957)
192. Tabor, H., Rosenthal, S. M., and Tabor, C. W., *J. Am. Chem. Soc.*, **79**, 2978 (1957)
193. Greene, R. C., *J. Am. Chem. Soc.*, **79**, 3929 (1957)
194. Hirsch, M. L., *Ann. inst. Pasteur*, **93**, 199–209 (1957)
195. Shive, W., *Ann. N. Y. Acad. Sci.*, **52**, 1212–34 (1950)
196. Dunn, F. W., *J. Biol. Chem.*, **227**, 575-81 (1957)
197. Aposhian, H. V., Wolff, S. M., Rhea, W. G., Jr., *Arch. Biochem. Biophys.*, **71**, 442–50 (1957)
198. Kuchinskas, E. J., Horvath, A., and Vigneaud, V. du, *Arch. Biochem. Biophys.*, **68**, 69–75 (1957)
199. Gots, J. S., *J. Biol. Chem*, **228**, 57–66 (1957)
200. Bolton, E. T., and Mandel, H. G., *J. Biol. Chem.*, **227**, 833–44 (1957)
201. Balis, M. E., Levin, D. H., Brown, G. B., Elion, G. B., Nathan, H. C., and Hitchings, G. H., *Arch. Biochem. Biophys.*, **71**, 358-66 (1957)

202. Camien, M. N., and Dunn, M. S., *Arch. Biochem. Biophys.*, **70**, 327-45 (1957)
203. Minami, K., *J. Bacteriol.*, **73**, 338–44 (1957)
204. Shaefer, W. B., *J. Bacteriol.*, **73**, 52–55 (1957)
205. Amos, H., and Vollmayer, E., *J. Bacteriol.*, **73**, 172–77 (1957)
206. Beaver, D. J., Roman, D. P., and Stoffel, P. J., *J. Am. Chem. Soc.*, **79**, 1236–45 (1957)
207. Beltz, R. E., and Visser, D. W., *J. Biol. Chem.*, **226**, 1035–45 (1957)
208. Sakurogi, T., and Kummerow, F. A., *Arch. Biochem. Biophys.*, **71**, 303–10 (1957)
209. Rakestraw, J. A., and Roberts, E. R., *Biochim. et Biophys. Acta*, **24**, 388–96 (1957)
210. Bullen, W. A., and Frear, D. S., *Arch. Biochem. Biophys.*, **66**, 502–3 (1957)
211. Takahashi, H., and Nason, A., *Biochim. et Biophys. Acta*, **23**, 433–35 (1957)
212. Hills, G. M., *Biochem. J.*, **45**, 353–62 (1949)
213. O'Brien, R. T., and Campbell, L. L., Jr., *J. Bacteriol.*, **73**, 522–25 (1957)
214. Hyatt, M. T., and Levinson, H. S., *J. Bacteriol.*, **74**, 87–93 (1957)
215. Murty, G. G. K., and Halvorson, H. O., *J. Bacteriol.*, **73**, 230–34 (1957)
216. Miller, L. P., and McCallan, S. E. A., *J. Agr. Food Chem.*, **5**, 116–22 (1957)
217. French, R. C., and Weintraub, R. L., *Arch. Biochem. Biophys.*, **72**, 235-37 (1957)
218. Gordon, M. A., *J. Bacteriol.*, **73**, 199-205 (1957)
219. Hitzman, D. O., Halvorson, H. O., and Ukita, T., *J. Bacteriol.*, **74**, 1–7 (1957)
220. Hughes, W. H., *Bacteriol Anatomy*, 341–60 (Cambridge University Press, Cambridge, England, 360 pp., 1956)
221. Spoerl, E., Sarachek, A., and Puchett, N., Science, **125**, 601 (1957)
222. Holden, J. T., and Holman, J., *J. Bacteriol.*, **73**, 592–93 (1957)
223. Rhuland, L. E., *J. Bacteriol.*, **73**, 778-83 (1957)
224. Meadow, P., Hoare, D. S., Work, E., *Biochem. J.*, **66**, 270–82 (1957)
225. Work, E., *Nature*, **179**, 841-47 (1957)
226. Sall, T., Mudd, S., Payne, J. I., *J. Bacteriol.*, **74**, 794–800 (1957)
227. Gavin, J. J., *Appl. Microbiol.*, **5**, 235–43 (1957)
228. Macias, F. M., *Appl. Microbiol.*, **5**, 249–52 (1957)
229. Deibel, R. H., Evans, J. B., Niven, C. F., Jr., *J. Bacteriol.*, **74**, 818–21 (1957)
230. Camien, M. N., Yuvilei, A., and Dunn, M. S., *Proc. Soc. Exptl. Biol. Med.*, **94**, 137–42 (1957)
231. Wolff, R. L., Dubost, S., and Brignon, J. J., *Proc. Soc. Exptl. Biol. Med.*, **95**, 270–72 (1957)
232. Seidler, A. J., Nayder, F., and Schweigert, B. S., *J. Bacteriol.*, **73**, 670-75 (1957)
233. Hoff-Jørgensen, E., *Biochem. J.*, **50**, 400–3 (1952)
234. Sneider, U. C., and Potter, R. L., *Proc. Soc. Exptl. Biol. Med.*, **94**, 798-800 (1957)
235. Guthrie, R., Loebeck, M. E., and Hillman, M. J., *Proc. Soc. Exptl. Biol. Med.*, **94**, 792–94 (1957)
236. Adelson, L. M., Schatz, A., Trelawny, G. S., *J. Bacteriol.*, **73**, 148–53 (1957)
237. Snell, E. E., *Trace Elements*, 547–74 (John Wiley and Sons, Inc., New York. N. Y., 672 pp., 1957)

# NUTRITION AND ECOLOGY OF PROTOZOA AND ALGAE[1]

By Luigi Provasoli[2]
*Haskins Laboratories, New York, New York*

## Introduction

Considerable interest has developed during the last few years in nutritional studies directed towards determining the factors responsible for blooming of protozoa and algae. This recourse to the laboratory in a search for relevant data was overdue and was stimulated by Lucas' enticing theory that in waters there are "non-predatory relationships based on the release and biological influence of metabolites, ranging from toxins to vitamins and hormones" (1, 2, 3). These metabolites are excreted or secreted by some organisms and become a significant part of the environment of other organisms.

Simultaneously, the nutritionists succeeded in replacing, with known vitamins, some empirical organic extracts needed by many algae and protozoa cultured axenically, thus lending experimental weight to Lucas' hypothesis. The obvious assumption that "if an organism requires a growth factor *in vitro,* this metabolite, or its physiological equivalent, should be found in significant amount in the environment" (4), became accepted and proved to be true.

The ecology of algae and protozoa, mainly concerned so far with the effect of the abiotic environment on the organism, is finally aligning itself with the realities of ever changing cyclic systems in which the effect of the abiotic environment on organisms, the effect of organisms on the environment, and the interactions of organisms all contribute to the biogeochemical cycles. The water environment is the one in which these effects can be expected to be most efficient because most of the biologically active molecules are water soluble and because the water environment is a continuous system in contrast to the discontinuity and patchiness of the rhizosphere and other land environments. We should be able, therefore, to detect more clearly the excretion and consumption of the metabolites which govern the interdependent growth of different groups of organisms.

What are the decisive parameters and how are we to detect and measure them? Field studies and data on the chemical composition of the abiotic environment (major salts and trace metals) are indispensable to the nutritionist for establishing raw laboratory cultures and, later, bacteria-free

[1] The survey of the literature pertaining to this review was concluded in November, 1957.

[2] Aided in part by contract NR 163-202 with the Office of Naval Research, by research grants G-3216 and B-1198 from the National Institutes of Health, and by Grant G-650 from the National Science Foundation.

cultures; media which mimic the natural environment as closely as possible have the best chances of success.

The next phase is to outline the nutritional requirements of the ecologically important organisms and to determine their idiosyncrasies—a study which is far more detailed than is normally the case in nutritional work. Is the organism photoautotrophic or heterotrophic (obligate, facultative, or ambivalent); are growth factors needed; what are the tolerances toward total solids, the preferences for salt ratios, special needs for trace elements, the favorable N, P, and C sources? These studies have already uncovered the effect of unsuspected metabolites which are present in nature in concentrations too low to be detected chemically (see section on vitamins and plant hormones p. 295). Besides identifying the effective metabolites it is necessary to know their complete range of action, including toxicity. Inhibition and toxicity data are extremely important in differentiating predominantly steno- or eurybionts from organisms which, while extremely sensitive (steno-) to some parameters, are tolerant (eury-) toward others.

It is often said that laboratory conditions are wholly artificial and that laboratory experiments at best vaguely approximate natural conditions. But the ecological arena is populated by the products of the continous challenge of nature to the potentialities of the organisms. Consider for a moment the extremes of thermal or arctic waters; the light of the tropics or the dimness of the arctic; dystrophic lakes, ponds, ditches, and mines, as compared to normal lakes, estuaries, brackish swamps, brine lagoons and internal seas (Dead Sea, Salton Sea), as compared to the oceans. In fact, the challenges provided by nature are as strenuous to the organisms as are those inflicted on them by extreme conditions in exploratory laboratory experiments.

After vast labors, ecologists have recognized some species as typical denizens of this or that environment. It is then logical to assume that "biological indicators" such as these are stenobionts at least for some conditions under study, and that the difficulty in finding reliable biological indicators resides principally in excluding the stenobionts which, because of their slightly wider flexibility toward some parameters, can colonize some overlapping environments. If so, indicator species need to be the first to be worked on by ecologically-minded nutritionists.

Laboratory data do not always correlate with field observations, but this, in pointing out pitfalls and lack of knowledge is valuable, too. A classical example of stretching laboratory data is the tenet "all algae are photoautotrophic" that has straitjacketed limnology and oceanography for years. The data which led to this idea had been derived almost exclusively from Chlorophyceae; furthermore, these species had been preselected because they had been isolated from mineral media. We know now that many algae and flagellates need vitamins and that the Chlorophyceae is the algal group with few auxotrophic species. One factor which led to awareness of the correct situation was the lack of correlation between the nitrate and phosphate content of natural waters and the growth of some algae. We assume

then that when laboratory data fail to explain ecological situations, an important parameter has been missed and more laboratory work is called for.

When dealing with specific ecological problems it is dangerous to extrapolate data from one species to another, even in the same genus, unless we know that many species of the genus behave alike. It would, actually, be best to isolate each species from the environment under study. Algae and protozoa comprise variants as do other groups of microorganisms!

The review centers on algae and algal flagellates. Ciliates have been reviewed elsewhere (5). Relationships between the phagotrophic organisms which involve prey-predator relationships do not, at this time, lend themselves to nutritional analysis.

## EFFECTS OF THE ABIOTIC ENVIRONMENT ON THE ORGANISMS

The presence and the success of an organism in the environment is controlled by the quality and quantity of metabolites; deficiency or excess or both may be limiting. As the environments fall into broad categories (oligosaprobe, mesosaprobe, etc.), the various algal groups also seem to display some general distinctive nutritional features. Therefore, some generalizations can be made.

For lakes, Pearsall (6) observed that diatoms prefer waters rich in N, P, and silica, with a monovalent ion/divalent ion ratio below 1.5, and Rao (7) found that they favor alkaline waters. Silica is often the limiting factor for diatoms; according to Lund (8) and Komarovsky (9), the decline of diatom populations results largely from lack of silica.

Chlorophyceae seem, as a group, the most resistant to a variety of conditions. Many species withstand, utilize, or need high concentrations of nutrients; fish ponds (9), (generally fertilized with an excess of organic or inorganic fertilizers), nutrient rich ponds [Singh (10)], and sewage oxidation ponds are generally dominated by green algae. Notwithstanding this, desmids and other Chlorophyceae occur in English lakes in summer and in waters poor in Ca, N, and P (6), and are scarce in waters high in $NH_4$ and low in $O_2$ (7).

Cyanophyceae thrive in neutral, and better in alkaline waters rich in oxidizable organic compounds; they can grow rapidly in minimal concentrations of N and P (6, 7).

Various euglenids and cryptomonads favor acid waters when Fe and the ratio C:N are high (7). Pigmented and colorless euglenids thrive in polluted ponds and barnyard pools extremely rich in organic matter, and their growth in fish ponds depends upon organic fertilization and disappearance of Cyanophyceae (9).

Each environment, in contrast, has species whose potentialities seem so closely fitted to their environment as to typify it. These biological indicators are more reliable and sensitive than chemical analysis [Fjerdingstad (11) and Foghammar (12)]. Fjerdingstad considers that diatoms alone are not

sufficient to characterize waters and gives a series of algal indicators for waters ranging from the catharobe to the polysaprobe (11). Rawson (13) discusses the advantages and disadvantages inherent in using phytoplankton quotients and dominant species for selecting biological indicators of trophic conditions of lakes, and proposes a list of indicators for Canadian lakes. As already mentioned, indicator organisms are the ones needing attention from nutritionists; unfortunately, most nutritionists are unfamiliar with ecological problems and an up-to-date comprehensive list of biological indicators, annotated by data on the chemical composition of the waters they represent, is needed to stimulate nutritional studies.

Klotter (14) employs a very clear graphic representation, the "milieu-chart." Each organism is depicted by a series of small charts, one for each variable of the environment: geographical distribution, temperature, pH, hardness, organic matter (as consumption of $KMnO_4$), Cl, $SiO_2$, Fe, Mn, $NH_4$, $NO_2$, $NO_3$, and $PO_4$. Data are lacking on Na, K, Mg, and Ca as well as on vitamins, Zn, Co, and Cu. A silhouette in each chart represents the relative quantity of growth for each month. Klotter gives data for 452 species of algae and flagellates of the lakes of the Black Forest; in addition to his own observations he has incorporated ecological records on individual algae from the literature. The charts could be improved by putting the relevant data on cards for automatic sorting machines. Although Klotter justly cautions that we may not yet know all the limiting factors, his method of charting, if widespread, will reveal where knowledge is needed. Klotter emphasizes the importance of describing the conditions of waters when phytoplanktonts are in initial logarithmic growth. These, not the ones found during blooms, are the real optimal conditions. Too many records, especially of the sea, are just occasional observations or a record of bloom conditions, and their value is limited.

The need for systematic, closely-spaced observations is emphasized by Bamforth (15). Complete measurements were done every other day and several correlations emerged; monthly samplings would have missed them completely. Such closely-spaced observations over a long period of time are absolutely essential, as Bamforth shows, especially for a limited natural environment (he observed a small pond). They are as necessary for larger environments although a little more widely spaced in time, because "the phytoplankton lives perilously close to minimal conditions and slight environmental changes can promote rapid growth, reduction, or subsidence of populations."

The ecologists could also start soil-water biphasic or other bacterized cultures of ecologically important organisms; this would be an excellent point of departure for nutritionists. Parke's collection of marine organisms and Pringsheim's more extensive collection have been extremely useful to the author and to many others.

In beginning studies on organisms from a new environment, the odds

for eventual success are greater if one does not begin with indicator organisms but rather with eurybionts which inhabit overlapping environments; they are more likely to be tamed in the laboratory and to withstand our clumsiness because of their resistance to a variety of conditions. The Chlorophyceae, some diatoms, and euglenids have thus become laboratory weeds, but the delicate oligotrophic species of fresh-water reservoirs yielded only to the patient efforts of Chu (16). The main nutritional characteristic of the oligotrophic forms was found to be their need for extremely low total solid concentrations. Chu, and Rodhe (17) were the pioneers in the field; they systematically explored the complete range of each nutrient and obtained optimal specific media for single organisms as well as media for general application. Thus, Chu's No. 10 has served to isolate and culture many blue-green algae [Gerloff, Fitzgerald & Skoog (18)]. It is also suitable, when conveniently enriched, for 3 species of *Synura* and a dinoflagellate *Woloszynskia* (Provasoli & Pintner, unpublished data). Rohde's No. VIII served for culturing several Chlorococcales, Volvocales, heterokonts, and desmids. Provasoli & Pintner (4) similarly developed media for *Synura petersenii* and *Cryptomonas ovata;* in a table they compare their media with Chu 10 and Rodhe VIII. The three mineral media are very similar and strikingly close to the standard ionic composition found by Rodhe for many Swedish lakes (19); common denominator media mimic natural waters in respect to major elements (the N and P requirements of the organisms *in vitro* are generally much higher than in natural waters).

Although planktonic fresh-water algae and flagellates share a need for low concentrations of total solids, they differ in the levels of nutrients needed and in their ratios. Few nutritional studies have directly sought ecologically pertinent information; nevertheless, scattered in the nutritional literature one finds perhaps significant idiosyncrasies. We will review a few with no pretense at completeness, but rather, with the hope of stimulating more awareness of the ecological implications of nutritional studies.

*Major elements.*—The features differentiating two typical extreme types of water—the calcareous-carbonate type of fresh water and sea water—are (*a*) total solids; (*b*) ratio of monovalent to bivalent ions; (*c*) predominant anions and cations; (*d*) ratio of Ca/Mg. Which of these parameters are the important ones for the organisms? How restricted are the organisms in respect to the inorganic constituents of their typical environment?

Pearsall (6), from ecological observations, considers that a monovalent/divalent ratio (M/D) below 1.5 is favorable to diatoms of oligotrophic waters. Chu (16) and Vollenweider (20) find experimentally that three oligotrophic diatoms, *Fragilaria, Asterionella,* and *Tabellaria* are all stimulated by high Ca, and that K interacts with Ca and Mg. This interaction is considered by Vollenweider as antagonistic while Chu interprets this to mean that excessive K renders these organisms tolerant to higher concentrations of divalent ions. This could also be interpreted as a preference for a certain

monovalent/divalent ratio; Provasoli, McLaughlin & Pintner (21) confirm the preference for Ca and find that *Fragilaria capucina* prefers an M/D ratio below 1.5. *Synura petersenii,* on the contrary, prefers low total solids concentrations (60 to 100 p.p.m.) and monovalent ions (M/D above 2); when these two conditions are met, wide variations in the Ca/Mg ratio are compatible with growth.

Vollenweider also considers the interaction of Ca and Mg in a wide range of concentrations. *Oscillatoria rubescens* and *Ankistrodemus falcatus* show an interesting time effect; in the first few days of growth, increasing Ca stimulates cell division and growth; this effect later fades and the density of final growth depends upon Mg concentration. Vollenweider observes that with Mg at suboptimal levels, the addition of Ca enhances growth, and vice versa. The same interchangeability of Ca and Mg at suboptimal levels is found by Vishniac (22) for the less stenohaline strain of a marine *Labyrinthula.*

Provasoli, McLaughlin & Pintner (21) studied the effect of various Ca and Mg concentrations, and Ca/Mg ratios (from 24:1 to 1:24) in relation to total solids and to different M/D ratios. The organisms studied have, in general, a wide flexibility toward different Ca/Mg ratios, which seems to narrow with unfavorable total solid concentrations and M/D ratios. At high M/D ratios (i.e., more monovalent ions) Ca may become an important ion, as for *Fragilaria capucina* when the M/D ratio is above 1.5. The same phenomenon seems to hold for marine organisms, but at much higher concentrations of NaCl, as for *Rhodomonas lens* (21) and for *Phaeodactylum tricornutum* [Droop (24)]. It was also found that brackish and marine littoral organisms can grow at quite different Ca/Mg ratios and need far less Ca and Mg than the environment offers them: one-half the sea water concentration of Mg and one-quarter the concentration of Ca elicit good growth. These findings enabled Provasoli, McLaughlin & Droop (23) to formulate chemically-defined marine media which do not precipitate on sterilization. This was achieved by (*a*) lowering Ca and Mg drastically; (*b*) employing less Ca than Mg (it has more soluble salts); (*c*) replacing phosphates with glycerophosphates (which have soluble heavy ion salts); and (*d*) adding metal chelators. Such media allow good growth of a variety of brackish, marine littoral and supralittoral algae and flagellates. Unpublished results on additional organisms indicate that, provided Ca and Mg are present in a minimal necessary quantity (which for Ca seems to vary from species to species), these organisms are quite tolerant to different Ca/Mg ratios. We concluded that Ca and Mg are widely interchangeable [(23) p. 104]. Droop (24) finds that four supralittoral euryhaline species (*Monochrysis lutherii, Hemiselmis virescens, Nannochloris oculata,* and *Phaeodactylum tricornutum*) are extremely tolerant to different Ca/Mg ratios; they have Ca and Mg optima independent of each other, and their optimal Ca/Mg ratio resembles neither the Ca/Mg ratio characteristic for fresh water nor for sea water. This tolerance and versatility gives them an eco-

logical advantage because rock pools are subjected to wide changes in salinity and M/D ratios through sea water sprays, desiccation, and rain.

The experiments of Droop show no interchangeability of Ca and Mg at the 1:10 concentration intervals shown in his diagrams, but at the crossing of the optimal concentrations of Mg and Ca there is a zone of near-optimal growth. Perhaps in this zone, and within a more limited range of concentrations, there is Mg—Ca interchangeability since most of the data of Provasoli, McLaughlin & Pintner (21) were obtained in a comparable zone and with more gradations in concentrations.

Droop (24) describes some characteristics of the astounding physiological potentials of these euryhaline species: *Monochrysis* is the most tolerant to all parameters; the optimal Mg requirement of *Nannochloris* is higher than the Mg content of sea water; *Phaeodactylum* shows interference between Ca and Na, and the Ca optimum rises with rising Na. Droop also reports that the marine neritic diatom, *Skeletonema costatum,* usually considered euryhaline, is distinctly stenohaline by comparison with the four supralittoral organisms. The oceanic *Rhodomonas lens* studied by Provasoli, McLaughlin & Pintner (21) is also stenohaline.

Droop concludes that because these organisms are indifferent to different M/D and Ca/Mg ratios, the most important factor, upon dilution of sea water, is Na; Na tolerance may be the decisive parameter which distinguishes among neritic, supralittoral, and estuarine species.

How much do NaCl tolerances or need, reflect an osmotic requirement or a specific need for Na? Experiments with the brine organism *Dunaliella salina* show that only 90 per cent of the NaCl requirement could be substituted for by other osmotically-active electrolytes [Hutner *et al.* (25)]. Allen & Arnon (26) find Na essential for the blue-green alga *Anabaena cylindrica;* this is the first proof of essentiality of Na for a plant. K, Li, Rb, and Cs cannot substitute for Na. Five p.p.m. of Na suffice for optimal growth and there is no evidence that larger amounts are harmful. It is likely that the sodium requirement is a general characteristic of the blue-green algae because Allen (27), with less exacting techniques, found that 23 cultures of various Cyanophyceae can grow in Na-containing media, without added K. Gerloff, Fitzgerald & Skoog (28) report a marked beneficial effect upon the addition of Na to cultures of *Microcystis aeruginosa,* and Kratz & Myers (29) find that logarithmic growth of *Anabaena variabilis, Anacystis nidulans,* and *Nostoc muscorum* cannot be maintained without Na. These observations may be significant ecologically because eutrophic lakes and lakes affected by civilization often have blooms of blue-green algae. Various factors, especially richness in utilizable organic products, have been thought to be responsible. Urbanization not only enriches the lakes with organic materials, although most of it should be eliminated by sewage treatment, but also with sodium and, in recent times, with phosphorus because of the increased use of detergents.

In discussing with Edmondson possible factors influencing the appearance

of blue-green algae blooms in Lake Washington, Seattle, we considered that the main difference before and after the appearance of *O. rubescens* blooms was the urbanization of the lake shore [Edmondson, Anderson & Peterson (30)] and the increase of salinity resulting from the opening of a canal to the sea. Because of the lock system, the increase of salinity is slight but probably significant; one wonders if increased Na, among other factors, is responsible for tipping the balance in favor of the blue-green algae.

Salinity (as dilution of the normal sea water) is an important ecological factor as shown by Nordli (31), who finds that the distribution and mass occurrences in the North Sea and near the coast of Norway of 3 species of *Ceratium* are controlled by salinity and temperature; the laboratory data on salinity and temperature accord with the ecological data. Similar studies of Braarud (32) and of Braarud & Pappas (33) show that the optimal salinity of many neritic North Sea species is 20 to 25 per thousand although these species are normally found in the coastal waters of Norway which have a salinity of 25 to 30 per thousand. *Rhodomonas* (21) and *Skeletonema* (24), and other neritic species isolated from sea water of 30 to 35 per thousand salinity also show a preference for a salinity of 20 to 24. This may indicate that one of the normal constituents of sea water is present in concentrations approaching inhibition. However, other species of neritic dinoflagellates isolated from the Carribean and the Gulf of Mexico (salinity 36 per thousand) dislike the low salinity (20 to 23 per thousand) of artificial media (23) and prefer artificial media quite similar in composition and salinity, to 33 per thousand (unpublished data, Provasoli & Gold). This may depend upon a different tolerance to NaCl, as Droop suggests and, indeed, the few data at our disposal indicate possible stepwise differences in salinity limits for neritic organisms (24, 31, 32, 33).

The oceanic species offer an even greater challenge: the diatom *Chaetoceros decipiens* can be cultured in enriched sea water media in the presence of bacteria but will not grow bacteria-free either in similar media or in artificial media. Since the artificial media are rich in all known B-vitamins, Provasoli, McLaughlin & Droop [(23) p. 420] consider that bacteria may be a stabilizing influence on the physical state of the medium. This might be true especially for diatoms. Lewin (34) found that *Navicula pelliculosa* cannot assimilate silica unless a compound containing divalent sulfur is added to the medium. Even in bacterized cultures Harvey (35, 36) found that a variety of organic sulfur compounds were required for vigorous growth of *Ditylum brightwellii,* and Matudaira (37) added organic sulfide to the list for *Skeletonema costatum.* Droop (38) shows that the need for divalent sulfur for *Skeletonema* depends on aeration. Vigorously aerated cultures require 3 mg. $Na_2S \cdot 9H_2O$ per liter while cultures inoculated into a freshly sterilized medium without aeration grow as well without added sulfide. This is also the case for the fresh-water diatom *Fragilaria*

*capucina;* aeration from the time of inoculation is definitely inhibitory. This inhibition is completely removed by the addition of 0.1 mg. per cent of cysteine·HCl: aeration started after 3-day growth does not inhibit (Provasoli & Pintner, unpublished data). Droop (38) concludes that "the interdependence of sulphur requirement and oxygen tension may be reflecting the lability of the thiol radical in redox systems . . . or may mean that the function of these thiols is to maintain a critical redox potential of the cell surface."

*Trace metals.*—Several trace metals are known to be essential for the growth of algae. The universal requirement for iron needs no comment. Pirson (39), in an excellent review, discusses the criteria of essentiality, the need and physiological role of iron, boron, sulfur, manganese, molybdenum, zinc, vanadium, and cobalt in algae and in higher plants. The data on algae were obtained mainly on *Chlorella, Scenedesmus,* and *Anabaena cylindrica;* so far Mo, Cu, Mn, V, and Co have been demonstrated to be essential for them and we may assume that this holds for all algae.

The maximum quantity needed and the toxicity of each element presumably vary in the different algal groups and possibly from species to species. Pillai (40), working with euryhaline blue-green algae, finds that concentrations of copper which inhibit *Phormidium tenue* actually stimulate *Spirulina* and *Oscillatoria* sp. For some elements, especially copper, the limits between need and toxicity may be extremely narrow. Copper at $10^{-7}M$ inhibits photosynthesis in *Chlorella* [Greenfield (41)], yet a series of poisons of copper catalysts inhibits photosynthesis in *Chlorella* [Green, McCarthy & King (42)] This apparent contradition is resolved by the findings of Walker (43) which indicate that at lower concentrations Cu shows up as an essential element for *Chlorella. Hydrodictyon* must be protected against the toxic effect of small quantities of heavy metals, especially copper, by additions of soil extract or other chelating agents [Neeb (44)]. Wilson & Collier (45) find that soil extract and sulfide improve the growth of *Gymnodinium breves,* and Wilson (personal communication) suggests that sulfide may be beneficial by precipitating Cu, an ion extremely toxic to *G. breves.*

As noted, no one has succeeded in cultivating true oceanic species in bacteria-free culture but some oceanic diatoms can be grown in bacterized cultures. It is unlikely that oceanic forms need more growth factors than the neritic species because growth factors are mainly produced by bacteria near the coasts; bacteria are scarce in open waters. But another difference between the two types of water may be important: sparse chemical data and a few biological hints indicate that coastal waters are richer in trace elements, probably from runoff from rivers and the proximity of soil and rocks. This may also apply to the large fresh-water lakes. Copper concentrations vary widely in the sea. Atkins (46) records variations from about 25 mg. per $m.^3$ in winter to 1.5 mg. per $m.^3$ in early autumn in surface waters 20 miles off shore in the English Channel. Kalle & Wattenberg (47) find less

than 3 mg. per m.$^3$ in mid-Atlantic waters, and Chow & Thompson (48) report 25 mg. per m.$^3$ in low salinity waters near the mouth of the Mississippi River and less than 1 mg. per m.$^3$ in other areas. Kylin, A. (49, 50) and Kylin, H. (51), employing germinating zygotes of *Ulva* and *Enteromorpha* as test organisms, proved that waters of the *Fucus-Ascophyllum* zone are rich in nitrates, phosphates, and trace metals, and that concentrations of these important elements diminish progressively with depth and distance from shore. Infertile waters at 30 m. depth and, strikingly, even more waters at 70 m. depth, became fertile when enriched with Zn, Mn, Fe, and Co. Harvey (52) concluded that inshore waters have enough Ni, Mo, V, Zn, and Cu because the addition of these ions did not affect the growth rate of a marine *Dunaliella* (referred to by Harvey as a *Chlamydomonas*). Many oceanic diatoms were cultured by Allen & Nelson (53) in their enriched sea water; enrichment causes abundant precipitate. The precipitate is essential for the effectiveness of the medium and this may arise from co-precipitation of substances present in sea water in concentrations nearing toxicity. This valuable hint was, however, overlooked by all of us because the Erd-Schreiber medium (a sea water enriched with soil extract which also forms a precipitate during sterilization) successfully replaced the old Allen & Nelson medium, especially for littoral organisms. We interpreted this to mean that soil extract was useful because of its abundant growth factors and trace metals. This approach proved to be extremely useful. Chelated mixtures of trace metals and shot-gun vitamin mixtures effectively substituted for soil extract and permitted the growth in defined media of fresh-water, brackish, and marine neritic organisms but failed to solve the problem of the oceanic forms. The aforementioned observation of Neeb (44) indicates that the humic acids in soil extract may be in excess and may serve as an exchange resin, releasing Ca and Mg and binding preferentially, Fe, Mn, Zn, Co, and Cu for which metal chelators have more affinity. Furthermore, about 50 per cent of the iron and manganese in sea water are in particulate form, the rest being in colloidal solution [Lewis & Goldberg (54)]. It is logical to suppose, then, that pelagic organisms, to obtain enough of these elements, developed powerful methods of trapping and dissolving trace metals and therefore might be oversensitive under laboratory conditions. Diatoms utilize particulate iron [Goldberg (55)]; seaweeds concentrate various trace metals in their cytoplasm from 10 to 10,000 times [Black & Mitchell (56)]; flagellates absorb strontium [Rice (57)]; and various fresh-water algae concentrate radioactive P from 100,000 to 800,000 times [Krumholz (58)]. Hutner *et al.* (59) postulated a competition between environmental chelators and cellular chelators; this led to the wide use in media of organic chelators which were effective in keeping trace metals in solution in the alkaline range and in withstanding heat sterilization. By balancing chelators and trace metal concentrations one can create a nontoxic reservoir of metals, thus allowing prolonged growth of fast-growing organ-

isms. But, while accumulation of trace metals and chelating agents suits some eurybionts (*Euglena, Ochromonas, Herpetomonas, Tetrahymena,* etc.), this does not please more sensitive brackish and marine neritic algae and flagellates. For them one has to balance metal chelators against trace metals and to overchelate between 1.5 and 3 times (23). Fresh-water planktonic algae and flagellates require very little trace metals, and if no chelators are added the impurities carried in by the chemically "pure" salts introduce enough trace metals except for Fe, and perhaps Mn, which can then be added as metal ammonium citrates to avoid precipitation. Better media for these sensitive organisms will be possible when purer salts become available.

While chelated media meet some desires of the nutritionists, i.e., more growth, more reproducible media, and more kinds of organisms in chemically defined media, they have not, so far, particularly furthered knowledge on the quantitative and qualitative need for single trace metals. At best, as noted, we can say that tolerance to, and probably the quantitative need for trace metals seem to parallel the general potentials of the organisms; needs are higher in eurybionts, especially those of meso- and polysaprobic waters, and lower in stenobionts. This trend is a valuable guide for reseach but has limited ecological use.

The addition of a metal chelator, e.g., ethylenediaminetetracetic acid (EDTA), brings the need for chelatable essential metals into the open. Need for a specific metal is demonstrable if a metal, less strongly chelated, overcomes the inhibitory effect of the chelator, e.g., if inhibition by EDTA is overcome by Ca, Ni, and Cu, calcium is needed and not vice versa [Hall (60)]. Additional experiments might enable a choice to be made between the possibilities that Ni or Cu are effective because they release Ca by displacing it from the metal complex, either through their greater affinity or by mass action, or because they are really required in addition to Ca. But better perspectives are offered. When *Euglena* and *Ochromonas* are grown at temperatures above normal, severe imbalances occur and the need for vitamins, purines, pyrimidines, amino acids, and metals rises dramatically [Baker, Hutner & Sobotka (61) and Hutner *et al.* (62)]. Hutner *et al.* (63) propose to combine the temperature effect with chelating agents to amplify the need of metals far above the impurities of the media, thus permitting easy identification of the essentiality of elements like V, Co, and Cu which are needed in extremely small amounts, and eventually to bring into the open the need for other ions not known so far to be essential for life. For organisms whose requirement for an element is higher than the eventual traces left after chemical purification of the media, the refined techniques of Arnon and the Wisconsin school have given the only ecologically applicable data because they are quantitative. The advent of sterilizable plastic culture vessels, plastic stills, anionic exchange resins, and the advances of analytical methods already offer the possibility of further purification of the mineral constituents of media, hence more precise data.

The difficulties of the nutritionist in the study of trace metals are shared by chemists interested in natural waters. Harvey (36) and Hutchinson (64), in their wonderful books, review the meager data on trace metals in sea water and in fresh water and discuss their role in fertility. The problem of analyzing trace metals in natural waters, especially in sea water, has been a difficult one. The new techniques of Carritt (65) permit routine analysis of a group of trace metals and it is hoped that they may become normal hydrological procedures. Carpenter (66) has extended this method to a more rapid and precise analysis of the major constituents.

*Temperature.*—A recent example shows the ecological importance of temperature in the competition among species. Conover (67) finds that *Skeletonema costatum* and *Thalassiosira nordenskioldii* are the two dominant diatoms in Long Island Sound at the end of the winter. In 1953, *S. costatum* became overwhelmingly dominant; in 1954, *T. nordenskioldii* became more important. In February, 1953, the mean water temperature was 3.2° and in 1954, 1.7°C. Laboratory competition experiments showed that *T. nordenskioldii* competes successfully with *S. costatum* at temperatures less than 2 to 3°C.

The work of Hutner and his collaborators (61, 62, 63), showing that temperature is, for practical purposes, a nutritional variable for flagellates lends to the ecological temperature data an entirely new meaning. The sequence of events which the rise of temperature induces in different flagellates and probably in algae, is not an isolated instance; data on flagellates parallel data on thermophilic bacteria and even, in some respects, on events in mammals during fever.

A main difference is that the elevation of half a degree Centigrade above the ordinarily lethal temperature for *Euglena* and *Ochromonas* (34 to 35°, respectively) induces a sequence of nutritional deficiencies, similar to that produced by a rise of a few degrees in thermophilic bacteria, which have a higher lethal temperature. The experiments show that the upper temperature "limit" can spell death or more rapid growth, depending upon the available nutrients. Therefore, in rich environments the possibility of survival is greater for thermophilic organisms (in which the thermal inactivation of enzymes is slower and spread over more degrees), less for organisms like *Euglena* and *Ochromonas,* and probably minimal for cold-water stenotherms. "Permeable" heterotrophs and phagotropic eurybionts living in polluted environments (rich in all sorts of nutrients) have a better chance of withstanding temperature excursions near the upper limit than organisms living in oligotrophic waters.

One of the dramatic effects of temperature in *Ochromonas* and *Euglena* is the increase in vitamin requirements; the vitamin $B_{12}$ requirement, for instance, may increase several hundredfold. Since many algae and flagellates require vitamins and most of them require $B_{12}$, the temperatures straddling the upper limit may become ecologically important. Requirements of vita-

mins for phytoplankton, to be valid for ecological considerations, should be determined in the laboratory at temperatures well below the upper temperature limit, and the comparison of habitats of a single species can be vitiated if we do not take into consideration the nutritional deficiencies caused by high temperatures; such effects should be most evident in auxotrophic forms.

The geographical range of a species may be influenced by the nutritional effect of temperature. Colonization of warmer waters by colder water forms may be possible in rich waters. The succession of forms in rock pools, mud flats, shallow ponds, marshes, and other environments subjected to the widest temperature fluctuations may be similarly affected, and if these waters are low in vitamins nonauxotrophic forms may have the advantage. These ecological speculations are perhaps premature; we need to know (*a*) do high temperatures induce new qualitative requirements for vitamins; (*b*) does the growth response toward a vitamin vary with temperature, and is it higher at lower temperatures within the normal temperature range of a species; (*c*) is there any nutritional effect of temperatures straddling the lower limit of tolerance of a species.

*Nitrogen.*—Nitrogen fixation is widespread in blue-green algae; according to Fogg (68), 21 species belonging to 8 genera have this ability. There is no good evidence to suggest that species of other algal groups and flagellates can fix nitrogen but not all the species available have been critically investigated for this ability.

The ecological and practical importance of nitrogen fixation is obvious (68). Several blue-green algae are endosymbiotic or have symbiotic relationships with fungi or higher plants. Winter (69) and Henriksson (70) report that *Nostoc* isolated from root parenchyma of *Gunnera* and from the lichen *Collema* (70) are nitrogen-fixers and release soluble nitrogen in culture media. Extracellular products in healthy cultures of blue-greens have often been reported; Fogg (71) finds that *Anabaena cylindrica* produces mostly polypeptides and only minute amounts of free amino acids. These polypeptides are not a nitrogen source for *A. cylindrica* or for *Chlorella*. The extracellular substances produced by the nostocs of lichens may be different or not, but the fungal partner can assimilate them. Bond & Scott (72), using $N^{15}$, find that two lichens and a liverwort containing *Nostoc* assimilate $N_2$. De (73), Singh (74), and Watanabe (75) find that the blue-green algae of rice fields fix nitrogen; various lines of evidence indicate that they are principally responsible for the sustained fertility of rice fields.

Most algae and photosynthetic flagellates utilize both nitrate and ammonium ions; ammonium is preferentially absorbed when both N-forms are present. Colorless flagellates, except *Polytoma ocellatum* [Lwoff & Dusi (76)], several euglenids: the *Euglena gracilis* group (Hutner, unpublished data), *Trachelomonas abrupta, T. pertyi,* and *Phacus pyrum* (Provasoli

& Pintner, unpublished data) cannot utilize nitrates. Nitrate is generally used in algal media in preference to ammonium because the uptake of ammonium generally lowers the pH of the poorly-buffered fresh-water media, inhibiting growth (phosphate buffering is often impossible because oligotrophic organisms are generally very sensitive to phosphates).

Furthermore, ammonium becomes rapidly toxic in alkaline media; some species are especially sensitive. Lysis of *Prymnesium parvum* by ammonium sulphate has been used by Reich & Aschner (77) to control the poisonous blooms of this organism in brackish fish ponds in Israel. But, in some cases, the treatment was ineffective.

Shilo & Shilo (78) found *in vitro* that lysis increases with temperature and decreases sharply with pH: 1 to 2 mg. per cent of $(NH_4)_2SO_4$ lyses at pH 8–9; at pH 7 more than 15 mg. per cent are needed. McLaughlin (79) confirms this and shows that ammonium is an excellent source of nitrogen below pH 7 and that toxicity decreases with salinity. Most euryhaline and marine flagellates studied in our laboratory show poor toleration for ammonium in alkaline media. The *in vitro* concentrations of ammonium are generally so high that $NH_4$ toxicity is unlikely to be ecologically important in normal saline environments but $NH_4$ toxicity may be significant in special environments, such as rock pools enriched by bird excreta or by decaying seaweeds, and in severely polluted bays.

Moriches Bay and Great South Bay sustained for years a prosperous oyster industry, but recently the oysters have almost disappeared. Concomitantly, the number of duck farms along the tributary streams had been increasing and the usual marine phytoplankton was replaced by blooms almost exclusively composed of *Nannochloris atomus* and *Stichococcus* sp. These chlorophytes were found by Ryther (80) to be well-fitted to their ecological niche: they utilize $NO_3$, $NH_4$, urea, uric acid, and amino acids at 1 mg. atom *N*/l. equally well; grow best at a N : P ratio of 5 : 1, within a salinity range of 3 to 34 per thousand at optimal temperatures of 15 to 25°C. A *Nitzschia* isolated from the same bay grew poorly in $NH_4$ and organic N, was stenohaline and preferred lower temperatures. In September, 1953, a hurricane reopened the partially sand-blocked inlets to the sea; two years later the blue point oyster reappeared. Similar fitness to a rich environment was found by Allen (81) in sewage oxidation ponds. Here, again, the green algae predominate in a succession: *Chlorella, Chlamydomonas, Scenedesmus,* governed by their nutritional idiosyncrasies.

*Phosphorus.*—Phosphorus and nitrogen are generally present in very low concentrations in nature; they are the most evident limiting factors. McCombie (82) and Hutchinson (64), in their reviews, tabulate and discuss the few laboratory data on planktonic fresh-water species. The work of Chu (83) and Rodhe (17) makes it evident that the optimal ranges for N and P vary from species to species. The results in our laboratory, though not as detailed, show a similar trend. Fresh-water species (*Synura, Wolo-*

*szynskia*), and brackish species (*Rhodomonas lens, Gyrodinium californicum, G. uncatenum, G. resplendens,* and *Gymnodinium splendens*) are photoautotrophic with no heterotrophy except for needing growth factors. For these species the optimal range for sodium or potassium nitrate is 3 to 10 mg. per cent and for phosphates 1 to 5 mg. per cent. Phototrophic species with heterotrophic abilities (*Trachelomonas abrupta, T. pertyi* and *Phacus pyrum*) have a higher optimal range and greater tolerance to higher concentrations of N and P. Species from polluted environments [*Prymnesium parvum* and *Monochrysis lutherii,* McLaughlin (79)] have an optimal range, respectively, of 1 to 100 mg. and 1 to 50 mg. per cent phosphate and 10 to 300 mg. per cent nitrate. The range of phosphorus allowing growth is far lower and narrower than for nitrogen and varies for different organisms. Stenobiosis and eurybiosis for N and P seem to correlate with the steno- or eurybiosis for other parameters. For the more homogenous environment of Swedish lakes, Rodhe (17) finds that the "content of P varies within the interval below 20 μg. P/l." From this finding, other field observations, and laboratory data on oligotrophic organisms, Rodhe divides phytoplanktonts into three categories in respect to phosphorus requirements: (*a*) low requirement: upper limit $< 20$ μg.; (*b*) medium requirement: lower limit $<$ and upper limit $> 20$; (*c*) high requirement: lower limit $> 20$ μg. P/l. However, the concentrations of N and P which produce optimal growth *in vitro* are generally between 10 to 100 times higher than the concentrations which produce similar growth in nature.

But, as Ryther (84) points out, blooms of organisms may also be produced by the concentration in space of the organisms themselves. Winds, zones of mixing and sinking of two water masses of different salinities, and convection water cells—all can concentrate the organisms in elongated patches or streaks, a pattern common in blooms. Therefore, the organisms had drawn their nutrients from a much larger volume of water, and this could explain in many cases the discrepancies between laboratory and field data. These concentrating mechanisms are greatly favored by tactisms of the organisms. Phototactical diurnal migrations of dinoflagellates were observed in detail by Halse (85, 86) for several marine dinoflagellates, and by Baldi (87) for a mountain *Glenodinium.* The author has seen the spectacular rise, from the bottom, of enormous clouds of these red glenodinia as soon as the sun rays passing between two mountain peaks illuminated the water directly; the blue-green water of the lake became streaked in a few minutes with red. In both cases the organisms migrate between the surface and depths of 5 to 10 m. Active migrations, and the variable buoyancy of diatoms, by themselves permit utilization of nutrients from a larger volume of water.

The discrepancy between the laboratory data and field observations with respect to phosphorus may have more subtle causes. Rodhe (17) found that the addition of 1 to 5 μg. P/l. to "bacteria-filtered" water of Erken Lake

stimulated full growth of *Asterionella* while no growth was obtained in artificial media with 9 to 18 μg. P/l. These results agree with those reported by Chu (83) and Lund (88). A P-sparing factor may be present in natural waters but Rodhe [(17), p. 80] did not advance far in the search for this factor because of erratic results obtained under apparently uniform experimental conditions.

It is also possible that some of the phosphate added to mineral media precipitates during sterilization, becoming unavailable; the proportion precipitated varies and depends upon the length and temperature of sterilization. This could explain why higher concentrations are needed *in vitro* and also the erratic results found by Rodhe and by us. Provasoli & Pintner (unpublished data) obtained at times with P-depleted inocula of *Fragilaria capucina* one-third optimal growth with 110 μg. inorganic phosphate P/l., and with 6 μg. glycerophosphate P/l. In one instance, the lower limit of growth was observable with 33 μg. inorganic P/l. and 0.6 μg. glycerophosphate P/l., but these experiments were erratic. As reported by Benoit (89), we thought at the time that organic P is more easily assimilated than inorganic P.

Provasoli, McLaughlin, and Pintner (unpublished data), extended the original findings of Chu (90), of Harvey (91) on glycerophosphate, and of Rodhe (17) on adenylic acid to many fresh water and saline organisms. Glycerophosphate, monoethylphosphate, guanylic, cytidylic, and yeast nucleic acids are utilized in different degrees by all the organisms tried; glycerophosphate is more effective for several species than inorganic P. McLaughlin (79) obtained full growth of *Prymnesium parvum, Monochrysis lutherii,* and *Isochrysis galbana* with 1.1 mg. inorganic P/l. and with 100 μg. glycerophosphate P/l. It is necessary to find out whether the glycerophosphate effect is specific or results from avoidance of precipitates during sterilization because of the higher solubility of Ca and Mg glycerophosphate.

In any event, since all the organisms tested utilized organic P, mineralization of P appears dispensible; indeed organic P may be more efficiently utilized. As a result, turnover of P may be speedier, compensating in part for the scarcity of this element. Another variable is the ability to concentrate phosphorus intracellularly. Krumholz (58) observed that in White Oak Lake, which receives low-level radioactive wastes from Oak Ridge, the algae and flagellates concentrate radioactive phosphorus in different degrees: *Euglena* about 100,000 times; *Volvox* 140,000; *Pandorina* 285,000; *Spirogyra* 850,000 times. The different levels of need and tolerance to P shown by algae and flagellates may reflect their enormous and variable power to concentrate P. Differences in tolerance to phosphorus, while not commonly ecologically important, may be of great importance in the pollution and fertilization of natural waters. In the latter case, the emphasis has been to obtain increasing productivity (as measured by chlorophyll content), with too little attention given to the qualitative changes in phyto-

plankton composition. From laboratory data one may expect that high concentrations of fertilizer will favor eurybionts, and low concentrations the normal phytoplanktonts, although, because of their sensitivity, it might be difficult to reach a balanced increase of nutrients which will favor them exclusively. Eurybionts seem to dominate in fish ponds, i.e., blue-green and green algae in fresh waters; in brackish waters the poisonous *Prymnesium* may often become dominant. Pratt (92) reports that *Prymnesium* sp. and *Nannochloris oculata* were two of the three dominant forms in tanks of fertilized sea water. The situation may also be found in nature as the aforementioned blooms of green algae in Great South Bay. Where pollution is not too high, as in Oslo Fjord, diatoms, dinoflagellates, and coccolithophorids may bloom spectacularly; some of these are normal coastal forms, but even the oceanic-Atlantic *Coccolithus huxleyi* blooms in the Fjord [Braarud (93)]. Similarly, the successful fertilization of Bare Lake, Alaska, by Nelson & Edmondson (94) which improved the weight of salmon, apparently did not destroy the sensitive algal species as shown by improved growth of *Asterionella* and *Tabellaria*.

*Vitamins and plant hormones.*—Droop (38) and Provasoli (95) review from an ecological point of view the need of marine flagellates and algae for vitamins. Provasoli (96), in considering some aspects of the morphological and physiological evolution of the algae, tabulates the vitamin requirements of fresh water and saline water species. Since this work appeared in an Italian journal of limited accessibility, the tables are repeated here (Table I) and enlarged to include data on thiamine requirements in saline flagellates and algae which Dr. Droop generously sent to this reviewer in manuscript; other friends likewise gave unpublished information. The botanical classification is adhered to because, according to available information, the colorless flagellates (i.e., leucophytic protozoa) came from pigmented counterparts. As J. Lederberg put it, streptomycin cures *Euglena* of its chloroplasts, becoming an *Astasia*—a taxonomically embarrassing event. Table I includes only those *Chlorophyceae* for which vitamin requirements have been specifically studied; many other unstudied species are in bacteria-free culture and most likely do not require vitamins, but elimination of chemical contaminations may reveal auxotrophy in a few. Most of the species in the Table are flagellates; the work of Pascher, Pringsheim, and Lwoff (97) emphasized the evolutionary loss of photosynthetic pigments, i.e., the transitions chlorophyte-saprophyte, and chlorophyte-animal. Some algae have predominant vegetal tendencies (e.g., *Chlorophyceae, Bacillariophyceae*); in others saprophytic or holozoic tendencies predominate (*Euglenineae, Cryptophyceae, Dinophyceae,* and *Chrysophyceae*). Although data on auxotrophy are scant, one correlation can be perceived: auxotrophy predominates in algal groups with well-developed saprophytic and holozoic tendencies and is weak in groups with strong vegetal tendencies. Auxotrophy does not correlate with energy source; it occurs in many

TABLE I

VITAMIN REQUIREMENTS

| Species | $B_{12}$(*l*) | Thiamine | Biotin | Reference |
|---|---|---|---|---|
| **CHLOROPHYCEAE** | | | | |
| *Astrephoneme gubernaculifera* (*a*) | O | R(?) | O | (99) Stein |
| *Brachiomonas submarina* | O | O | O | (100) Droop |
| *Chlamydomonas aglöeformis* | O | O | O | (101) Lwoff |
| *Chlamydomonas chlamydogama* (*b*) | R | O | O | (102) Hutner & Provasoli |
| *Chlamydomonas moewusii* | O | O | O | (102) Hutner & Provasoli |
| *Chlamydomonas reinhardii* | O | O | O | Smith (Personal communication) |
| *Chlorella vulgaris* | O | O | O | |
| *Chlorogonium elongatum* | O | O | O | (103) Loefer |
| *Chlorogonium euchlorum* | O | O | O | (103) Loefer |
| *Coelastrum morus* (?) | O | R | O | (104) Lewin |
| *Dunaliella salina* | O | O | O | |
| *Dunaliella primolecta* | O | O | O | Provasoli (Unpublished data) |
| *Dunaliella viridis* | O | O | O | (105) Gibor |
| *Gonium pectorale* | R | O | O | (99) Stein |
| *Haematococcus pluvialis* | O | O | O | (101) Lwoff |
| *Lobomonas pyriformis* | O | O | O | (106) Osterud |
| *Lobomonas rostrata* | R | O | O | (104) Lewin |
| *Nannochloris atomus* | O | O | O | (80) Ryther |
| *Nannochloris oculata* | O | O | O | (100, 121) Droop |
| *Pilinia* sp. | O | O | O | Lewin (Unpublished data) |
| *Platymonas* sp. | O | O | O | (105) Gibor |
| *Polytoma caudatum** | O | R | O | (76) Lwoff & Dusi |
| *Polytoma obtusum** | O | O | O | (76) Lwoff & Dusi |
| *Polytoma ocellatum** | O | R | O | (76) Lwoff & Dusi |
| *Polytoma uvella** | O | O | O | (76) Lwoff & Dusi |
| *Polytomella caeca** | O | R | O | (76) Lwoff & Dusi |
| *Prasiola stipitata* | O | O | O | (107) Lewin |
| *Prototheca zopfii** | O | R | O | (108) Anderson |
| *Pyramimonas inconstans* | R | R | O | Lewin (Unpublished data, confirmed by Provasoli & Gold) |
| *Selenastrum minutum* (Cambridge Culture Collection) | O | O | O | (104) Lewin |
| *Selenastrum minutum* | O | R | O | (104) Lewin |
| *Stephanoptera gracilis* | O | O | O | (105) Gibor |
| *Scenedesmus obliquus* | O | O | O | |
| *Stichococcus cylindricus* (?) | O | O | O | (80) Ryther |
| *Stichococcus cylindricus* (?) | R | O | O | (128) Lewin |
| *Stichococcus* sp. | O | O | O | (105) Gibor |

* Colorless species.

(*a*) May use *para*-aminobenzoic acid.

(*b*) Requires histidine.

| Species | $B_{12}$(*l*) | Thiamine | Biotin | Reference |
|---|---|---|---|---|
| *Volvulina steinii* (*a*) | O | R(?) | O | (99) Stein |
| **EUGLENINEAE** | | | | |
| *Astasis longa** (=*A. klebsii*, VonDach) | R | R | O | (109) Thayer |
| *Euglena gracilis* var.: *typica, bacillaris, urophora* | R | R | O | (110) Hutner *et al.* |
| *Euglena gracilis** permanently bleached (*c*) | R | R | O | (111) Robbins *et al.* |
| *Euglena pisciformis* | O | R | O | (112) Dusi |
| *Euglena stellata* | R | R | O | Hutner *et al.* (Unpublished data) |
| *Euglena viridis* | R | R | O | Hutner *et al.* (Unpublished data) |
| *Peranema trichophorum** (*d*) | R | R | O | (113) Storm & Hutner |
| *Phacus pyrum* | R | R | O | Provasoli & Pintner (Unpublished data) |
| *Trachelomonas abrupta* (*e*) | R | S | O | (114) Provasoli & Pintner |
| *Trachelomonas pertyi* (*e*) | R | S | O | Provasoli & Pintner (Unpublished data) |
| **CRYPTOPHYCEAE** | | | | |
| *Chilomonas paramoecium** | O | R | O | (76) Lwoff & Dusi |
| *Cryptomonas ovata* (var. *palustris*) | R | O | O | (4) Provasoli & Pintner |
| *Cyanophora paradoxa* | R | O | O | (4) Provasoli & Pintner |
| *Hemiselmis virescens* (*f*) | R | R | O | (100, 121) Droop |
| *Rhodomonas lens* (*g*) | S | R | O | Provasoli & McLaughlin (Unpublished data) |
| *Rhodomonas* sp. (6 strains) | R | R | O | Provasoli & Gold (Unpublished data) |
| **DINOPHYCEAE** | | | | |
| *Amphidinium klebsii* (?) | R | R | R | (115) McLaughlin & Provasoli |
| *Amphidinium rhynchocephalum* (?) | R | R | R | (115) McLaughlin & Provasoli |
| *Exuviaella cassubica* | R | O | O | (116) Provasoli & McLaughlin |
| *Glenodinium foliaceum* | R | O | O | (121) Droop |
| *Gonyaulax polyedra* (*h*) | R | O | O | (117) Haxo & Sweeney |
| *Gymnodinium breves* (*i*) | R | R | R | (45) Wilson & Collier |
| *Gymnodinium splendens* | R | O | O | (118) Sweeney |

(*c*) Streptomycin-bleached.
(*d*) Also needs riboflavin. May need other vitamins.
(*e*) Thiamine not indispensable; necessary for prolonged good growth.
(*f*) Glycine is required.
(*g*) The addition of $B_{12}$ allows optimal growth.
(*h*) May need other vitamins.
(*i*) Other unknown requirements. Other vitamins in media not determined as essential.

| Species | $B_{12}$(*l*) | Thiamine | Biotin | Reference |
|---|---|---|---|---|
| *Gyrodinium californicum* (*Gyrodinium* sp.) | R | O | O | (4) Provasoli & Pintner |
| *Gyrodinium cohnii* (*j*) | O | S-R | R | (119) Provasoli & Gold |
| *Gyrodinium resplendens* | R | O | O | (116) Provasoli & McLaughlin |
| *Gyrodinium uncatenum* | R | O | O | (116) Provasoli & McLaughlin |
| *Peridinium balticum* | R | O | O | (116) Provasoli & McLaughlin |
| *Peridinium chattoni* | R | O | O | (116) Provasoli & McLaughlin |
| *Peridinium trochoideum* | R | O | O | (121) Droop |
| *Woloszynskia limnetica* (*Peridinium* sp.) | R | O | O | (4) Provasoli & Pintner |
| **CHRYSOPHYCEAE** | | | | |
| *Hymenomonas* (Syracosphaera) *carterae* | R | O | O | (4) Provasoli & Pintner |
| *Hymenomonas* (Syracosphaera) *elongata* | R | R | O | (120, 121) Droop |
| *Isochrysis galbana* | R | R | O | McLaughlin, Gold & Provasoli (Unpublished data) |
| *Microglena arenicola* | R | R | O | (120, 121) Droop |
| *Monochrysis lutherii* | R | R | O | (120, 121) Droop |
| *Ochromonas danica* | O | R | R | (122) Heinrich |
| *Ochromonas malhamensis* | R | R | R | (123) Hutner, Provasoli & Filfus |
| *Pleurochrysis scherffelii* (*k*) | O | R | O | Provasoli & Gold (Unpublished data) |
| *Poteriochromonas stipitata* | R | R | R | (123) Hutner, Provasoli & Filfus |
| *Prymnesium parvum* | R | R | O | (120, 121), Droop; (79) McLaughlin |
| *Stichochrysis immobilis* | O | O | O | Provasoli & Gold (Unpublished data) |
| *Synura caroliniana* | R | O | (?) | Provasoli & Pintner (Unpublished data) |
| *Synura petersenii* | R | O | O | (4) Provasoli & Pintner |

(*j*) *G. cohnii* has been carried through 20 transfers in biotin alone, but growth reaches only 60,000 cells/ml., while in biotin plus thiamine it reaches 500,000 cells/ml. Histidine is necessary, but it can grow without histidine after adaptation.

(*k*) Another chrysomonad with filamentous stages and coccolithophorid zoospores needs only thiamine.

(*l*) $B_{12}$: organisms responding to cyanocobalamin (i.e., "true" $B_{12}$ = antipernicious anemia factor).

R = required; S = stimulatory; O = not needed; S-R = borderline case, see (*j*); (?) = unconfirmed data.

| Species | $B_{12}(l)$ | Thiamine | Biotin | Reference |
|---|---|---|---|---|
| **BACILLARIOPHYCEAE** | | | | |
| *Asterionella formosa* | O | O | O | Provasoli & Pintner (Unpublished data) |
| *Amphora perpusilla* | R | O | O | (124) Hutner & Provasoli |
| *Fragilaria capucina* | O | O | O | Provasoli & Pintner (Unpublished data) |
| *Navicula pelliculosa* | O | O | O | (125) Lewin, J. |
| *Nitzschia putrida** | O | O | O | (124) Hutner & Provasoli |
| *Phæodactylum tricornutum* (*N. closterium* v. *minutissima*) | O | O | O | (126) Hutner |
| *Skeletonema costatum* | R | O | O | (127) Droop |
| *Stephanopyxis turris* | R | O | O | Provasoli & Gold (Unpublished data) |
| *Tabellaria flocculosa* | O | O | O | Provasoli & Pintner (Unpublished data) |

obligate phototrophs (*Chlamydomonas, Cryptomonas, Gyrodinium, Rhodomonas, Synura, Woloszynskia*) as well as in colorless obligate heterotrophs (*Astasia, Chilomonas, Polytoma, Peranema*), but some colorless heterotrophs need no vitamins (*Polytoma obtusum, P. uvella*). Fortunately for ecologists, algae and flagellates have a rather stereotyped pattern of need for vitamins; only cobalamin, thiamine, and biotin are required, singly or in various combinations. "This stereotyped behavior, despite the pitifully small sampling, seems too strange to be fortuitous if one considers the multiplicity of available metabolites in nature; it might express a physiological evolutionary tendency peculiar to the algae, especially those of the chlorophyte-leucophyte transition" (96). The phagotrophic species and true protozoa [*sensu*, Lwoff (97)], seem to need more growth factors and "building blocks," i.e., preformed metabolites [Johnson (98)].

Auxotrophy does not correlate with any particular environment, which confronts us with provocative new parameters of uncertain ecological importance. Since the cobalamins are glamorous and two algae are practical assay organisms for $B_{12}$ in body fluids and natural crudes [e.g., Hutner, Bach & Ross (129)], attention has been lavished on cobalamins, but biotin and especially thiamine may be as important; cobalamins, nonetheless, seem required by more species than are thiamine and biotin. In order to judge whether vitamin $B_{12}$ may be limiting in nature, Provasoli (95) tabulated the few data on the sensitivity of algae to $B_{12}$. The sensitivity, i.e., the smallest amounts giving increased growth compared to the control without $B_{12}$, varies depending upon the organisms from 0.1 mμg./l. to 5mμg./l. Droop (130) brought the sensitivity of *Monochrysis lutherii* from the

original 2 to 3 mμg./l. down to 0.1 mμg./l. By knowing the volume of the cells and the number of flagellates obtained with definite amounts of $B_{12}$, he finds that about three molecules of $B_{12}$ are needed per $\mu^3$ of living *Monochrysis;* by recalculating the sensitivity data obtained by others for *Euglena* and *Stichococcus,* he obtains values of the same order. Applying this figure, he then reviews the few data on the content in vitamin $B_{12}$ of sea water and concludes that even the lowest amount found by Cowey (131)—0.1 mμg./l.—should support a crop of 25 millions of *Skeletonema* (the highest recorded from nature). Hence, from this calculation, vitamin $B_{12}$ should, at all times, be sufficient in the sea. Daisley (132), in an accompanying discussion, opposes this conclusion, pointing out that in nature the rate of cell-division is more important than the total yield obtainable with a given amount of $B_{12}$ because a certain rate of growth is necessary to build up a bloom despite losses from predation, sinking, etc. We might add that growth rate also figures in the competition of species for $B_{12}$. Daisley also remarks that Cowey extracted $B_{12}$ from filtered water with cyanide, a procedure known to liberate $B_{12}$ from binding substances. Since bound $B_{12}$ cannot be utilized by osmotrophs but could be used by phagotrophs, more $B_{12}$ is available for some species than for others, creating a differential.

The present data are scanty indeed but Droop's view seems correct for the coastal waters which apparently are richest in $B_{12}$. Kashiwada, Kakimoto & Kawagoe (133) find that the $B_{12}$ content of the fertile Kagoshima Bay varies with depth and time of the day; it fluctuates from zero to 55 mμg./l. at the surface in the inner Bay, and from zero to 5 mμg./l. at the entrance to the open sea. This accords with the isolated observations of Lewin (128) and of Droop (134) who found values between 5 to 10 mμg./l. for coastal waters; the earlier high values of Provasoli & Pintner (135) of 30 to 200 mμg./l. were found in waters of Woods Hole which had not been preserved at the moment of collection and are therefore misleading. Kashiwada *et al.* (136) map the content of 12 stations in the North Pacific along longitude 130°E and find mostly values varying from zero to below 1 mμg./l., thus supporting the data of Cowey. The significance of Cowey's finding is that $B_{12}$ content in the northern North Sea and the Atlantic dips from 2 mμg./l. in March to 0.3 mμg./l. in May-June. The dip coincides with the period of growth of the phytoplankton. $B_{12}$ may be an expensive but effective way for Co-starved organisms to acquire Co. Holm-Hansen, Gerloff & Skoog (137) found that 75 mμg./l. of $B_{12}$ in a Co-free medium give as much growth as 200 to 400 mμg./l. Co. This would make $B_{12}$ valuable even to organisms not requiring exogenous $B_{12}$, especially in localities very low in Co.

Analysis of $B_{12}$ in fresh water can be done routinely with the *Eugena* and *Ochromonas* assays which are rapid and precise (129, 138), but these organisms cannot tolerate sea water. Lewin (128) employed *Stichococcus* and Droop (134) *Monochrysis;* both organisms grow too slowly for convenience.

Cowey (131) reduces the salts and concentrates $B_{12}$ by phenol-ether extraction and assays the concentrate with *Lactobacillus leichmannii* and *Ochromonas*. Provasoli & Pintner (135) and, independently, Kashiwada *et al.* (136), simply dialyze sea water and assay the dialysate with *Euglena;* according to both, dialysis for 3 to 15 hr. does not lose $B_{12}$ appreciably (below 10 per cent) and allows desalting. Several laboratories are searching for euryhaline, heterotrophic $B_{12}$-requiring organisms to permit direct assaying of sea water.

However, many microorganisms can utilize numerous natural analogues of $B_{12}$ [Ford & Hutner (139)]. Saline (38, 95) and fresh-water algae (Provasoli & Pintner, unpublished) are no exception. Some, like *Ochromonas,* utilize only "true" $B_{12}$ (cyanocobalamin), $B_{12}$ III, and two benzimidazole-containing artificial analogues. Others, like *Euglena,* utilize also pseudo-$B_{12}$, Factors A and C; still others, like *Escherichia coli* 113–3 and *Skeletonema* utilize all cobalamins, even Factor B. It is therefore suggested that analysis of water be done with two organisms; one able to employ all cobalamins (total cobalamin) and one specific for true $B_{12}$ (95).

Long ago, Hutchinson (140) and Hutchinson & Setlow (141) sought and found thiamine, biotin, and nicotinic acid in fresh water. No record on this point exists for sea water; however, unidentified substances favoring growth of phytoplanktonts were extracted from sea water by Johnston (142).

Growth of *Ulva lactuca* germlings in bacteria-free culture is affected by plant hormones. In recent experiments a combination of kinetin and adenine permitted normal morphogenesis [Provasoli (143)]. Since seaweeds grow well in nature one would not expect plant hormones to be the limiting factors in coastal waters; however, suboptimal concentrations may affect speed of growth and total yield.

### Effects of the Organisms on the Abiotic Environment and Interactions Between Organisms

Algae affect the environment by removing nutrients, by excreting biologically active substances, and, after death, by restoring to the environment what they have removed and synthesized. A good example is the cobalamin cycle in natural waters. The producers, so far as is known, are bacteria, algae, and perhaps seaweeds; the direct consumers are other bacteria and algae, protozoa, and perhaps invertebrates. Robbins, Hervey & Stebbins (144) find that bacteria are the producers of $B_{12}$ in pond waters; Ericson & Lewis (145) and Starr, Jones & Martinez (146) find that 70 per cent of the marine bacteria produce larger quantities of cobalamin analogues than of cyanocobalamin itself (145). More bacteria make *L. leichmannii*-active cobalamins than *Euglena*-active cobalamins (146). Most water bacteria live epiphytically on particulate matter; suspended solids in sea water and brackish muds are found by Burkholder & Burkholder [(147), also a personal communication] to be relatively rich in analogues and poor in cyanocobalamin. Therefore, algae less specific towards cobalamins

should have an advantage, but data are meager. We need to know the ratios of total cobalamin to true $B_{12}$ in different environments and the specific needs of the ecologically significant organisms. Robbins, Hervey & Stebbins (148), Ericson & Lewis (145), and Hashimoto (149) find that generally the red seaweeds are richer in cobalamins than the brown seaweeds and that more species of red than brown contain cobalamins. Apparently the seaweeds absorb these vitamins from the environment (145), but the possibility is not excluded that some seaweeds may synthesize cobalamins because several blue-green algae and *Chlorella* synthesize cobalamins in $B_{12}$-deficient media (148, 149, 150). Blue-green algae, being abundant, may be an important source of $B_{12}$ in fresh water. Benoit (151) finds that in Linsley Pond the proportion of total cobalt tied up in $B_{12}$ is 13 per cent in the epilimnion and 2 per cent in the hypolimnion. He suggests that synthesis of the vitamin by blue-green algae in the photic zone is more efficient than that of bacteria in deeper waters. In sea water the blue-green algae are not particularly abundant except for the well-known blooms of *Trichodesmium erythraeum* but we do not know whether or not this species produces $B_{12}$ or requires $B_{12}$, as does *Phormidium persicinum*, another red pigmented marine blue-green alga [Pintner & Provasoli (152)]. Perhaps in the sea, production of $B_{12}$ centers around bacteria, which thus connects $B_{12}$ production with the supply of utilizable substrates; decaying of blooms should often result in a rise in $B_{12}$ content. Bacterial growth may also be favored by organic substances excreted by algae, a loose nutritional symbiosis. Indeed, some algae excrete between 20 to 40 per cent of the total organic matter photosynthesized [cf., e.g., Lewin (153, 154); Allen (155)]. It is probable, but not certain, that the mucilagenous polysaccharides observed by Lewin and others are utilized. Filtrates of *Chlamydomonas* promoted the growth of *Chlorella* in situations where *Chlorella* could not otherwise grow [Allen (81)].

Excreta of algae may serve other purposes. For instance, the polypeptides excreted abundantly by *Anabaena* are good solubilizers of tricalcium phosphate, making more P available, and they are also good chelators [Fogg & Westlake (156)]. The yellow pigments found in concentrations up to 10 mg./l. in fresh waters is a mixture of carboxylic acids which similarly chelate metals, keeping them soluble in alkaline waters [Shapiro (157)]. The yellow substances are among the few organic products which seem to accumulate in waters as does humic acid in soil, which implies that they are poor substrates. Most of the nutritionally important compounds have been found in waters but in such small amounts as to seem biologically unimportant; the available data are reviewed comprehensively by Vallentyne (158), and some interrelations between organic substances and phytoplankton are discussed by Saunders (159). The small amounts of organic substances in waters are misleading because, as noted, the algae secrete this material abundantly; if we do not find more organic substances it is because

they are utilized as rapidly as they are released into the water. Unialgal bacteria-free cultures offer the opportunity of identifying the products of algal metabolism because products pile up and are not consumed by other organisms. Zehnder (160) demonstrates that symbiotic algae are a source of thiamine for their fungal partner in lichens and Gäumann & Jaag (161) show that several unicellular soil algae produce the thiamine needed by phytopathogenic fungi.

The theory of Lucas (1) on interrelationships between organisms mediated by external metabolites has stimulated work in this area. Talling (162) finds that in nature competition between *Asterionella formosa* and *Fragilaria crotonensis* is not an antagonism mediated through external metabolites since filtrates, or mixed cultures, do not show such interactions. Lefèvre, Jakob & Nisbet (163) confirm older scattered findings, and show that many algae produce autoantagonists, and heteroantagonists. Their results were obtained mainly with filtrates of unialgal cultures, but filtered waters from algal gloom gave similar results. These substances are heatlabile and nothing is known about their composition.

The autoantagonistic chlorellin was a mixture of photoöxidized unsaturated fatty acids [Spoehr *et al.* (164)]. Proctor (165) reviews the literature in the most recent paper on algal heteroantagonism. Studying the interaction of five fresh-water species, he finds some species compatible, others cross-inhibitory; inhibition varies from mild to complete. In a detailed study of *Chlamydomonas reinhardii—Haematococcus pluvialis* interaction he finds that the inhibitory substance produced by *Chlamydomonas* is fat-like, soluble in alkaline media, steam-distillable from acidified solutions, and released upon death. He suggests that the inhibitor is probably a long-chain fatty acid or a mixture of them. This seems reasonable because palmitic and linoleic acids, he found, are very toxic for *Haematococcus*. The epilimnion of this problem has scarcely been pierced.

## LITERATURE CITED


1. Lucas, C. E., *Biol. Revs. Cambridge Phil. Soc.*, **22**, 270–95 (1947)
2. Lucas, C. E., *Symposia Soc. Exptl. Biol.*, **No. 3**, 336–56 (1949)
3. Lucas, C. E., in *Papers in Marine Biology and Oceanography*, 139–48 (Pergamon Press, Ltd., London, W.1, Engl., 498 pp., 1955)
4. Provasoli, L., and Pintner, I. J., *Ann. N.Y. Acad. Sci.*, **56**, 839–51 (1953)
5. Kitching, J. A., in *Microbial Ecology*, 259–86, 7th Symp. Soc. Gen. Microbiol. (Williams, R. E. O., and Spicer, C. C., Eds., Cambridge Univ. Press, Cambridge, England, 388 pp., 1957)
6. Pearsall, W. H., *J. Ecol.*, **20**, 241–62 (1932)
7. Rao, C. B., *J. Ecol.*, **41**, 62–71 (1953)
8. Lund, J. W. G., *J. Ecol.*, **38**, 1–35 (1950)
9. Komarovsky, B., *Bull. Research Council Israel*, **2**, 379–410 (1953)
10. Singh, R. N., *Revue Algologique*, **1**, 43 (1954)
11. Fjerdingstad, E., *Folia Limmologica Scand.*, **No. 5**, 1–123 (1950)
12. Foghammar, S., *Rapports Congr. Intern. Botan., 8th Congr.*, **Sect. 17**, 175–77 (1954)
13. Rawson, D. S., *Limnology and Oceanography*, **1**, 18–25 (1956)
14. Klotter, H. E., *Arch. Hydrobiol.*, **22**, 106–252 (1955)
15. Bamforth, S. S., *Ecological studies on the planktonic Protozoa of a small pond.* (Doctoral thesis, Univ. of Pennsylvania, Philadelphia, Pa., 1957)
16. Chu, S. P., *J. Ecol.*, **30**, 284–325 (1942)
17. Rohde, W., *Symbolae Botan. Upsalienses*, **10, 1–149 (1948)**
18. Gerloff, G. C., Fitzgerald, G. P., and Skoog, F., *Am. J. Botany*, **37**, 216–18 (1950)
19. Rodhe, W., *Proc. Intern. Assoc. Theoret. Appl. Limnology*, **10**, 377–86 (1949)
20. Vollenweider, R. A., *Schweiz. Z. Hydrol.*, **12**, 194–262 (1950)
21. Provasoli, L., McLaughlin, J. J. A., and Pintner, I. J., *Trans. N.Y. Acad. Sci.*, Ser. II, **16**, 412–17 (1954)
22. Vishniac, H. S., *J. Gen. Microbiol.*, **12**, 455–63 (1955)
23. Provasoli, L., McLaughlin, J. J. A., and Droop, M. R., *Arch. Mikrobiol.*, **25**, 392–428 (1957)
24. Droop, M. R., *Verhandl. Intern. Ver. Limnologie*, **13**, 722–30 (1958)
25. Hutner, S. H., Sanders, M., McLaughlin, J. J. A., and Scher, S., *Ann. N.Y. Acad. Sci.*, **69**, 286–91 (1957)
26. Allen, M. B., and Arnon, D. I., *Physiol. Plantarum*, **8**, 653–60 (1955)
27. Allen, M. B., *Arch. Mikrobiol.*, **17**, 34–53 (1952)
28. Gerloff, G. C., Fitzgerald, G. P., and Skoog, F., *Am. J. Botany*, **39**, 26–32 (1952)
29. Kratz, W., and Myers, J., *Am. J. Botany*, **42**, 282–87 (1955)
30. Edmondson, W. T., Anderson, G. C., and Peterson, D. R., *Limnology and Oceanography*, **1**, 47–53 (1956)
31. Nordli, E., *Blyttia*, **11**, 16–18 (1953)
32. Braarud, T., *Physiol Plantarum*, **4**, 28–34 (1951)
33. Braarud, T., and Pappas, I., *Avhandl. Norske Videnskaps-Akad. Oslo I. Mat.-Naturv. Kl.*, No. 2, 1–23 (1951)
34. Lewin, J. C., *J. Gen. Physiol.*, **37**, 589–99 (1954)
35. Harvey, H. W., *J. Marine Biol. Assoc. United Kingdom*, **23**, 499–519 (1939)

36. Harvey, H. W., *The Chemistry and Fertility of Sea Water* (University Press, Cambridge, Engl., 224 pp., 1955)
37. Matudaira, T., *Proc. Imp. Acad. (Tokyo)*, **18**, 107–16 (1942)
38. Droop, M. R., *J. Gen. Microbiol.*, **16**, 286–93 (1957)
39. Pirson, A., *Ann. Rev. Plant Physiol.*, **6**, 71–114 (1955)
40. Pillai, V. K., *Proc. Indo-Pacific Fisheries Council*, [II] **5**, 78–81 (1954)
41. Greenfield, S. S., *Am. J. Botany*, **29**, 121–31 (1942)
42. Green, L. F., McCarthy, J. F., and King, C. G., *J. Biol. Chem.*, **128**, 447–53 (1939)
43. Walker, J. B., *Arch. Biochem. Biophys.*, **46**, 1–11 (1953)
44. Neeb, O., *Flora*, **139**, 39–95 (1952)
45. Wilson, W. B., and Collier, A., *Science*, **121**, 394–95 (1955)
46. Atkins, W. R. G., *J. Marine Biol. Assoc. United Kingdom*, **31**, 493–94 (1953)
47. Kalle, K., and Wattenberg, H., *Naturwissenschaften*, **26**, 630–31 (1938)
48. Chow, T. J., and Thompson, T. G., *J. Marine Research (Sears Foundation)*, **11**, 124–38 (1852)
49. Kylin, A., *Kgl. Fysiograf. Sällskap. i Lund Förh.*, **13**, 185–92 (1943)
50. Kylin, A., *Kgl. Fysiograf. Sällskap. i Lund Förh.*, **16**, No. 5 (1945)
51. Kylin, H., *Kgl. Fysiograf. Sällskap. i Lund Förh.*, **16**, 225–29 (1946)
52. Harvey, H. W., *J. Marine Biol. Assoc. United Kingdom*, **26**, 562–79 (1947)
53. Allen, E. J., and Nelson, E. W., *J. Marine Biol. Assoc. United Kingdom*, **8**, 421–74 (1910)
54. Lewis, G. J., and Goldberg, E. D., *J. Marine Research (Sears Foundation)*, **13**, 183–97 (1954)
55. Goldberg, E. D., *Biol. Bull.*, **102**, 243–48 (1952)
56. Black, W. A. P., and Mitchell, R. L., *J. Marine Biol. Assoc. United Kingdom*, **30**, 575–84 (1952)
57. Rice, T. R., *Limnology and Oceanography*, **1**, 123–38 (1956)
58. Krumholz, L. A., *U.S. Atomic Energy Commission Document, Technical Information Service, ORO-132*, 1–54 (1954)
59. Hutner, S. H., Provasoli, L., Schatz, A., and Haskins, C. P., *Proc. Am. Phil. Soc.*, **94**, 152–70 (1950)
60. Hall, R. P., *J. Protozool.*, **1**, 74–79 (1954)
61. Baker, H., Hutner, S. H., and Sobotka, H., *Ann. N.Y. Acad. Sci.*, **62**, 349–76 (1955)
62. Hutner, S. H., Baker, H., Aaronson, S., Nathan, H. A., Rodriguez, E., Lockwood, S., Sanders, M., and Petersen, R. A., *J. Protozool.*, **4**, 259–69 (1957)
63. Hutner, S. H., Aaronson, S., Nathan, H. A., Baker, H., Scher, S., and Cury, A., in *Wooster Conference on Trace Metals* (Academic Press, Inc., New York, N.Y.; in press)
64. Hutchinson, G. E., *Geography, Physics, and Chemistry (A Treatise on Limnology, I)*, (John Wiley & Sons, Inc., New York, N.Y., 1015 pp., 1957)
65. Carritt, D. E., *Anal. Chem.*, **25**, 1927 (1953)
66. Carpenter, J. H., *Limnology and Oceanography*, **2**, 271–80 (1957)
67. Conover, S. A. M., *Bull. Bingham Oceanographic Collection*, **15**, 62–112 (1956)
68. Fogg, G. E., *Ann. Rev. Plant Physiol.*, **7**, 51–70 (1956)
69. Winter, G., *Beitr. Biol. Pflanz.*, **23**, 295 (1935)

70. Henricksson, E., *Physiol. Plantarum*, **4**, 542–45 (1951)
71. Fogg, G. E., *Proc. Roy. Soc.* (*London*), [B]**139**, 372–97 (1952)
72. Bond, G., and Scott, G. D., *Ann. Botany* (*London*), **19**, 67–77 (1955)
73. De, P. K., *Proc. Roy. Soc.* (*London*), [B]**127**, 121–38 (1939)
74. Singh, R. N., *Indian J. Agr. Sci.*, **12**, 743–56 (1942)
75. Watanabe, A., *Arch. Biochem. Biophys.*, **34**, 50–55 (1951)
76. Lwoff, A., and Dusi, H., *Compt. rend. soc. biol.*, **127**, 53–55 (1938)
77. Reich, K., and Aschner, M., *Palestine J. Botany, Jerusalem Ser.*, **4**, 14–23 (1947)
78. Shilo, M., and Shilo, M., *Proc. Intern. Assoc. Theoret. Appl. Limnology*, **12**, 233–40 (1955)
79. McLaughlin, J. J. A., *J. Protozool.*, **5**, 75–81 (1958)
80. Ryther, J. H., *Biol. Bull.*, **106**, 198–209 (1954)
81. Allen, M. B., *Calif. State Water Pollution Control Board Publ.*, **No. 13**, 1–48 (1955)
82. McCombie, A. M., *J. Fisheries Research Board, Can.*, **10**, 253–82 (1953)
83. Chu, S. P., *J. Ecol.*, **31**, 8–148 (1943)
84. Ryther, J. H., in *The Luminescence of Biological System*, 387–414 (Johnson, F. H., Ed., Am. Assoc. Advancement Sci., Washington, D.C., 452 pp., 1955)
85. Halse, G. R., *Oikos*, **2**, 162–75 (1950)
86. Halse, G. R., *Nytt Magasin Botanikk*, **2**, 139–47 (1954)
87. Baldi, E., *Mem. museo storia nat. ven. Tridentina*, **6**, 1–297 (1941)
88. Lund, J. W. G., *J. Ecol.*, **37**, 389–419 (1949)
89. Benoit, R. J., *Sewage and Ind. Wastes*, **27**, 1267–69 (1955)
90. Chu, S. P., *J. Marine Biol. Assoc. United Kingdom*, **26**, 285–95 (1946)
91. Harvey, H. W., *J. Marine Biol. Assoc. United Kingdom*, **31**, 475–76 (1953)
92. Pratt, D. M., *J. Marine Research (Sears Foundation)*, **9**, 29–54 (1950)
93. Braarud, T., *Proc. Intern. Assoc., Theoret. Appl. Limnology*, **12**, 811–13 (1955)
94. Nelson, P. R., and Edmondson, W. T., *U.S. Fish Wildlife Serv., Fishery Bull., No. 102* (1955)
95. Provasoli, L., in *Perspectives in Marine Biology* (Buzzati-Traverso, A. A., Ed., Univ. California Press, Los Angeles, Calif., in press)
96. Provasoli, L., *Boll. zool. agrar. e. bachicolt., Univ. studi Milano*, **22**, 143–88 (1956, but printed in 1957)
97. Lwoff, A., *L'Evolution Physiologique* (Hermann & Cie., Paris, France, 308 pp., 1943)
98. Johnson, W. H., *Ann. Rev. Microbiol.*, **10**, 193–212 (1956)
99. Stein, J. R., *Phycological News Bull.*, **32**, 74–75 (1957)
100. Droop, M. R., *J. Marine Biol. Assoc. United Kingdom*, **34**, 233–45 (1955)
101. Lwoff, A., *Recherches biochimiques sur la nutrition des protozoaires* (Masson et Cie., Paris, France, 158 pp., 1932)
102. Hutner, S. H., and Provasoli, L., in *Biochemistry and Physiology of Protozoa*, **I**, 27–125 (Lwoff, A., Ed., Academic Press, Inc., New York, N.Y., 434 pp., 1951)
103. Loefer, J. B., *Biol. Bull.*, **66**, 1–6 (1934)
104. Lewin, R. A., *Phycological News Bull.*, **5**, 21–22 (1952)
105. Gibor, A., *Biol. Bull.*, **11**, 223–29 (1956)

106. Osterud, K. L., *Physiol. Zoöl.*, **19**, 19–34 (1946)
107. Lewin, R. A., *Can. J. Botany*, **33**, 5–10 (1955)
108. Anderson, E. H., *J. Gen. Physiol.*, **28**, 287–96 (1945)
109. Thayer, P., *Studies on the nutrition* of Astasia klebsii *in synthetic medium* (Doctoral thesis, Amherst College, Amherst, Mass., 1949) [Cited in Kidder, G. W., *Ann. Rev. Microbiol.*, **5**, 144–56 (1951)]
110. Hutner, S. H., Provasoli, L., Stokstad, E. L. R., Hoffmann, C. E., Belt, M., Franklin, A. L., and Jukes, T. H., *Proc. Soc. Exptl. Biol. Med.*, **70**, 118–20 (1949)
111. Robbins, W. J., Hervey, A., and Stebbins, M. E., *Ann. N.Y. Acad. Sci.*, **56**, 818–30 (1953)
112. Dusi, H., *Compt. rend. soc. biol.*, **130**, 419–21 (1939)
113. Storm, J., and Hutner, S. H., *Ann. N.Y. Acad. Sci.*, **56**, 901–9 (1953)
114. Provasoli, L., and Pintner, I. J., *Phycological News Bull.*, **8**, 7–8 (1955)
115. McLaughlin, J. J. A., and Provasoli, L., *J. Protozool.*, **4**, (Suppl.), 7 (1957)
116. Provasoli, L., and McLaughlin, J. J. A., *J. Protozool.*, **2** (Suppl.), 10 (1955)
117. Haxo, F. T., and Sweeney, B. M., in *The Luminescence of Biological Systems*, 415–20 (Johnson, F. H., Ed., Am. Assoc. Advance. Sci., Washington, D.C., 452 pp., 1955)
118. Sweeney, B. M., *Am. J. Botany*, **41**, 821–24 (1954)
119. Provasoli, L., and Gold, K., *J. Protozool.*, **4** (Suppl.), 7 (1957)
120. Droop, M. R., *Nature*, **174**, 520 (1954)
121. Droop, M. R., *J. Marine Biol. Assoc. United Kingdom* (in press, 1958)
122. Heinrich, H. C., *Naturwissenschaften*, **14**, 418 (1955)
123. Hutner, S. H., Provasoli, L., and Filfus, J., *Ann. N. Y. Acad. Sci.*, **56**, 852–62 (1953)
124. Hutner, S. H., and Provasoli, L., *Phycological News Bull.*, **6**, 7-8 (1953)
125. Lewin, J. C., *J. Gen. Microbiol.*, **9**, 305–13 (1953)
126. Hutner, S. H., *Trans. N. Y. Acad. Sci.*, **10**, 136–41 (1948)
127. Droop, M. R., *J. Marine Biol. Assoc. United Kingdom*, **34**, 229–31 (1955)
128. Lewin, R. A., *J. Gen. Microbiol.*, **10**, 93–96 (1954)
129. Hutner, S. H., Bach, M. K., and Ross, G. I. M., *J. Protozool.*, **3**, 101–12 (1956)
130. Droop, M. R., *Nature*, **180**, 1041–42 (1957)
131. Cowey, C. B., *J. Marine Biol. Assoc. United Kingdom*, **35**, 609–20 (1956)
132. Daisley, K. W., *Nature*, **180**, 1042–43 (1957)
133. Kashiwada, K., Kakimoto, D., and Kawagoe, K., *Bull. Japan. Soc. Sci. Fisheries*, **23**, 450–53 (1957)
134. Droop, M. R., *J. Marine Biol. Assoc. United Kingdom*, **34**, 435-40 (1955)
135. Provasoli, L., and Pintner, I. J., *Proc. Soc. Protozoologists*, **4**, 10 (1953)
136. Kashiwada, K., Kakimoto, D., Morita, T., Kanazawa, A., and Kawagoe, K., *Bull. Japan Soc. Sci. Fisheries*, **22**, 637–40 (1957)
137. Holm-Hansen, O., Gerloff, G. C., and Skoog, F., *Physiol. Plantarum*, **7**, 665–75 (1954)
138. Ford, J. E., *Brit. J. Nutrition*, **7**, 299–306 (1953)
139. Ford, J. E., and Hutner, S. H., *Vitamins and Hormones*, **13**, 101–36 (1955)
140. Hutchinson, G. E., *Arch. Biochem.*, **2**, 143–50 (1943)
141. Hutchinson, G. E., and Setlow, J. K., *Ecology*, **27**, 13–33 (1946)
142. Johnston, R., *J. Marine Biol. Assoc. United Kingdom*, **34**, 185–95 (1955)

143. Provasoli, L., *Biol. Bull.*, **113**, 321 (1957)
144. Robbins, W. J., Hervey, A., and Stebbins, M. E., *Bull. Torrey Botan. Club*, **77**, 423–41 (1950)
145. Ericson, L. E., and Lewis, L., *Arkiv Kemi*, **6**, 427–42 (1953)
146. Starr, T. J., Jones, M. E., and Martinez, D., *Limnology and Oceanography*, **2**, 114–19 (1957)
147. Burkholder, P. R., and Burkholder, L. M., *Limnology and Oceanography*, **1**, 202–8 (1956)
148. Robbins, W. J., Hervey, A., and Stebbins, M. E., *Bull. Torrey Botan. Club*, **78**, 363–75 (1951)
149. Hashimoto, Y., *J. Vitaminol. (Osaka)*, **1**, 49–54 (1954)
150. Brown, F., Cuthbertson, W. F. J., and Fogg, G. E., *Nature*, **177**, 188 (1956)
151. Benoit, R. J., *Limnology and Oceanography*, **2**, 233–40 (1957)
152. Pintner, I. J., and Provasoli, L., *J. Gen. Microbiol.*, **18**, 190–97 (1958)
153. Lewin, R. A., *Biol. Bull.*, **109**, 373 (1955)
154. Lewin, R. A., *Can. J. Microbiol.*, **2**, 665–72 (1956)
155. Allen, M. B., *Arch. Mikrobiol.*, **24**, 163–68 (1956)
156. Fogg, G. E., and Westlake, D. F., *Proc. Intern. Assoc. Theoret. Appl. Limnology*, **12**, 219–32 (1955)
157. Shapiro, J., *Limnology and Oceanography*, **2**, 161–79 (1957)
158. Vallentyne, J. R., *J. Fisheries Research Board, Can.*, **14**, 33–82 (1957)
159. Saunders, G. W., *Botan. Rev.*, **23**, 389–410 (1957)
160. Zehnder, A., *Ber. schweiz. botan. Ges.*, **59**, 201–67 (1949)
161. Gäumann, E., and Jaag, O., *Phytopathol. Z.*, **17**, 218–28 (1950)
162. Talling, J. F., *Physiol. Plantarum*, **10**, 215–23 (1957)
163. Lefèvre, M., Jakob, H., and Nisbet, M., *Ann. Sta. Hydrobiol. Appl.*, **4**, 5–198 (1952)
164. Spoehr, H. A., Smith, J., Strain, H., Milner, H., and Hardin, G., *Carnegie Inst. Wash. Publ. No. 586*, 67 pp. (1949)
165. Proctor, V. W., *Limnology and Oceanography*, **2**, 125–39 (1957)

# BACTERIAL GENETICS[1]

By Arnold W. Ravin[2]
*The University of Rochester*
*Rochester, N.Y.*

It is barely possible to review in a detailed, yet meaningful, way a year's progress in the study of the heredity of microorganisms. The number and variety of investigations being pursued today are a tribute to the fruitfulness of the concepts and working hypotheses which have stimulated workers in this field. They have made the work of the annual reviewers increasingly difficult, however, and have led to the practice of dissecting the general field of microbial genetics into more manageable areas, usually on the basis of broad taxonomic groups, so that the genetics of viruses, of bacteria, of yeasts and molds, and of protozoa are dealt with separately. In keeping with this helpful practice, the genetics of bacteria will be emphasized in this review, but insofar as they are relevant to and shed light upon the problems under discussion, results with other microorganisms, and even higher organisms, will be considered.

This review is not presented as an exhaustive survey of the literature. Many reports have been omitted, in part by design and in part, very likely, by inadvertent neglect. The reviewer has sought to organize a fairly large body of papers governed by a few guiding principles; in doing so personal interests and views have had a great deal to do with the final selection and arrangement. In general, articles reviewed in the preceding volume (32) will not be considered here, unless more recent work requires their reconsideration. With few exceptions, no papers published after December, 1957, will be included in the discussion.

This review will consider four main topics: I. Mutation and replication of genetic material; II. Transfer of genetic material; III. Function of genetic material; and IV. Evolution of genetic material.

## MUTATION AND REPLICATION OF GENETIC MATERIAL

### Mutation

The term "mutation" has both a broad definition as well as a fairly delimited one. In the traditional and broad sense a mutation refers to a heritable modification. In addition to direct alteration of chromosomal structure, however, there are a variety of ways in which heritable changes can

[1] The following abbreviations are used: ADP (adenosinediphosphate); ATP (adenosinetriphosphate); DNA (deoxyribonucleic acid); RNA (ribonucleic acid); HFT (bacteriophage obtained from heterogenetic bacteria and transducing bacterial genetic determinants at high frequency); Hfr (bacterial donor strain transmitting in conjugation a group of genetic determinants at high frequency).

[2] Research of the author is supported by Grant E-727 awarded by the National Institute of Infectious and Allergic Diseases.

be brought about; for example, loss of photosynthetic ability in green flagellates by the destructive action of streptomycin on the chlorophyll-synthesizing system of the chloroplasts (177); loss of, or damage to, the cytochrome-synthesizing system in cytoplasmic granules (possibly mitochondria) of yeast (57); gain or loss of the "killer" character in *Paramecium* as a result of the cytoplasmic acquisition or depletion, respectively, of K-particles (201). Some new cases of nonchromosomal mutations have been reported recently. There is an interesting case studied by Spärck (202) in the mold *Neurospora crassa,* in which stable antigenic differentiation can be controlled through temperature in a way very similar to that described in *Paramecium* (8). Hanson (93) has described a spontaneous mutation in the K-particle itself, which affects the reproductive capacity of that particle. Novick & Wiener (168) have pointed out the close resemblance to mutation of induced enzyme synthesis at low concentrations of the inducer (as in induced β-galactosidase synthesis). Induced bacteria and their progeny continue to make enzyme at a maximal rate, provided a maintenance concentration of inducer is present, and, again like mutation, induction is the result of a random single event at the level of the cell. The induced system of enzyme synthesis decays, however, in the absence of a so-called maintenance level of inducer, and in this respect differs from a chromosomal mutation. In many respects, the switching on and off of enzyme synthesis by a substrate is like the environmental control of antigen synthesis so well studied by Sonneborn & Beale in *Paramecium* (8). The point to emphasize, of course, is that the stability of a particular metabolic state is the result of the interaction between chromosomal structure, the capacity of cytoplasmic structures, and environmental conditions (57a).

Quadling & Stocker (178) have studied the occurrence of rare motile bacteria in nonmotile strains of *Salmonella.* The presence of such apparent "mutants" in an otherwise nonmotile clone was inferred by the production of satellite microcolonies around the main growing mass of nonmotile bacteria plated on semisolid medium. Such satellites, in previous experiments involving transduction of the motility character (209), had been observed to consist of the sisters of abortively transduced motile bacteria (see discussion of Transduction in Section II). The rare motile bacteria in the nonmotile strain used in the present study were successfully isolated by micromanipulation. These motile cells proved to be ephemeral in their motility, for they gave rise to progeny nearly all of which were nonmotile. They did transmit motility to a few of their descendants, as shown by isolation with the micromanipulator of daughters in succeeding generations. The rare occurrence of motility could be explained as a result of the sporadic production (perhaps in the cytoplasm) of motility-conferring particles which were unable to reproduce in the absence of the proper genome but were transmitted to the progeny of cell division. Unable to reproduce and of limited number within the cell in which they are produced, these rare particles are thus conceived to be diluted rapidly among the progeny of succeeding gen-

erations. In one particular *Salmonella* strain studied, the frequency of the event giving rise to these hypothetical particles was calculated to be *ca.* $4 \times 10^{-5}$ per bacterium per generation. This frequency, although constant in a given strain, varied from one strain to another, thus indicating the probability that such an event is genetically determined. This work showed that an important contribution to heterogeneity in clones of microorganisms is made not only by the process of mutation itself, but also by "phenotypic excursions" within individual cells, the frequency of which is also under the control of the genotype.

Insofar as chromosomal changes are concerned, heritable modifications, broadly speaking, are not limited to localized chemical alterations of chromosomal structure (so-called "point" mutations), but may be brought about by changes in ploidy, by inversions of gene sequences in a chromosome, or by translocations of gene sequences from one chromosome to another (so-called "gross chromosomal aberrations"). A possible case of a camphor-induced polyploid strain of *Escherichia coli* (169) will be discussed in the next section. Unless otherwise indicated, we shall be concerned here with the narrower definition of mutation, with what may be termed "gene mutations." These are alterations of fairly restricted sites in the nuclear genetic material, which lead to rather specific consequences in the biochemical activities of the cell. Unfortunately, it is not always possible to demonstrate which of these various mechanisms is operating in the case of mutations in bacteria, unless, by recombination experiments, the locus in question can be shown to be part of a linear system of linked genes and thus, presumably, part of the nuclear apparatus of the cell. As yet, with present knowledge, it is the exception, rather than the rule, that such recombination experiments can be performed in bacteria. The discovery of the phenomenon of conjugation (in the K12 strain of *E. coli*) made possible the best known demonstration of linkage of bacterial genes (131). To some extent, as discussed in the next section, adequate demonstration of genetic linkage has been made with other bacterial species through the discovery of other methods of interbacterial gene transfer, such as transformation and transduction. It is only a fair presumption at the present time, however, that all bacteria possess a nuclear or chromosomal apparatus consisting of a linear array of linked genes. Cytological observations suggest, at least, that many bacterial species possess the Feulgen-positive chromatinic structures (or Robinow bodies) which behave in some respects like the chromosomes of higher organisms (184). Witkin (223) showed that certain mutations in *E. coli* segregated in a manner identical to that of the chromatinic structures; hence evidence not bearing directly on the linkage relationships of genetic determinants supports the assumption that the genes of bacteria are organized in DNA-containing structures. In any case, for most of the mutations discussed below, we must assume that they are the results of localized damage to the chromosomal structures.

The evidence for the assumption that the genetic information of the cell

nucleus resides in the deoxyribonucleic acid fraction will not be reviewed here. For a thorough discussion see (107). Suffice it to say that no recent evidence is in disharmony with this view which has been based on the well-known stability of DNA and its manner of replication (to be discussed below), the chemical nature of bacterial transforming agents (104), and the 1:1 relation of ploidy to DNA content (212). On the likely assumption that the nuclear genetic material consists of DNA, the questions arise: What is the physicochemical mechanism of gene mutation? What is the series of events that leads to a gene mutation, and how is the chemical structure of the gene altered in mutation? An especially promising approach has been that of Witkin (224), who has studied the influence of postirradiation metabolism on the expression of ultraviolet-induced mutations to prototrophy in *Salmonella typhimurium* and *E. coli*. She found that the yield of prototrophs is determined irreversibly by the amount of nutrient broth in the culture medium during the first hour of postirradiation treatment. A pool of amino acids had the same influence as nutrient broth; neither the presence of the growth factor required by the parental auxotroph alone nor a mixture of vitamins or purines and pyrimidines had any effect. The number of induced prototrophs increased, roughly, as the number of different amino acids in the postirradiation medium increased. Further pieces of evidence suggested strongly that the yield of prototrophs is proportional to the rate of protein synthesis during the first hour of postirradiation incubation, under conditions where protein synthesis is growth-limiting. First of all, prior adaptation of the auxotroph to growth in a minimal medium (containing limiting amounts of the required growth factor) gave rise to a greater number of prototrophs following irradiation than did preirradiation growth in nutrient broth. Second, the length of the lag phase in the postirradiation medium could be controlled through the amino acid enrichment level, and it was found, for three different auxotrophic strains, that the percentage of the maximum yield of induced mutants was a linear function of the length of the lag phase. Third, the use of a specific inhibitor of protein synthesis, chloramphenicol, during the postirradiation period showed that, under conditions which would otherwise produce a maximum yield of induced prototrophs, the presence of chloramphenicol led to a large reduction in the number of mutants obtained, with no significant effect on total survival. The effect of chloramphenicol was exhibited only during the first hour of incubation, exposure to the antibiotic after this time having no effect on the yield of prototrophs. Finally, if other metabolic activities besides protein synthesis were inhibited (during, for example, suspension in buffer without nutrients), the ability of irradiated auxotrophs to produce prototrophs was maintained for a very long period of time.

What is the significance of the period of postirradiation incubation during which the yield of mutants is sensitive to the availability of amino acids or exposure to chloramphenicol? The sensitive period is one hour when protein synthesis appears to be limiting growth, but not necessarily when

temperature, for example, is the limiting factor. The period of susceptibility to the postirradiation temperature, for a large variety of mutations, is a constant fraction (about one-third) of the lag phase (224). These facts indicate that two distinct processes are at work in gene mutation: (*a*) a process independent of the rate of protein synthesis, which determines the duration of the sensitive period, and (*b*) a process that is very strongly affected by the rate of protein synthesis. Preliminary measurements by Kanazir (121) of the rate of DNA synthesis in the irradiated auxotrophs used by Witkin shows that the amount of cellular DNA is doubled in approximately one-third of the lag period (1 hr. at 37°C.). It is tempting to suggest, therefore, as Witkin points out, that the process defining the duration of the susceptible period is that of DNA duplication. Protein synthesis might be expected to modify the yield of mutants by repairing lethal chromosomal damage (225, 226) and increasing the probability of mutant survivors. Upon the completion of DNA duplication, however, it would have to be supposed that the lethal damage was "fixed" and refractory to repair, at least by mechanisms connected to protein synthesis.

The effect of postirradiation protein synthesis may result, on the other hand, from the complex interrelation between protein synthesis and nucleic acid synthesis, as shown by Spiegelman and his colleagues (203, 204). If the synthesis of a new DNA molecule, incorporating a mutant (nonparental) component, is dependent upon concomitant protein synthesis, the facts could be equally well explained.

In the foregoing discussion, it is presumed that the site of ultraviolet damage is in the chromosomal DNA. This presumption is based upon a number of lines of evidence. Kanazir & Errera (122) have established that, in *E. coli* B and B/r, ultraviolet irradiation has the greatest relative inhibitory effect on DNA synthesis. At doses killing 80 per cent of the cells, neither growth, RNA, nor protein synthesis are immediately stopped, but DNA synthesis is blocked for a definite length of time. During this time probable nucleotide precursors of DNA accumulate. The idea that radiation damage is localized in chromosomal DNA is further supported by the work of Cohen & Barner. Utilizing a strain of *E. coli* (15 T−) which requires thymine for survival, they have shown (37) that deprivation of thymine to this strain results in "unbalanced growth" in which DNA, but not RNA, or protein, synthesis is halted. In recent experiments, for example, (6), they isolated four different mutants of the thymineless strain, each requiring for growth an exogenous supply of a different amino acid. It was observed that an amino acid deficiency sharply increased the survival of the strain in the absence of thymine. In the simultaneous presence of thymine and the required amino acids, death was very rapid. A comparison was made of the rate of death induced in this strain by ultraviolet irradiation and by the deprivation of thymine (5). These workers took advantage of the observation that incubation, without multiplication, in liquid media considerably restored the irradiated bacteria. Restored cells, on continued incubation

in liquid medium under conditions permitting growth, then proceeded to die at a rate similar to cells which were allowed to grow in the absence of thymine. Moreover, like thymineless death, ultraviolet-induced lethality is completed in the time necessary for a single cycle of multiplication. Like thymineless death, which was previously shown to be prevented by inhibiting other syntheses besides that of DNA (38), the secondary death of restored cells could be prevented by such inhibitors of cell metabolism as carbon or nitrogen depletion or treatment with 5-methyltryptophan.

Experiments of Iverson & Giese (113, 114) have also produced results consistent with the idea of DNA as the site of ultraviolet radiation damage. In studies of the effect of the various wave lengths on inhibition of RNA and DNA synthesis in *E. coli* B (113), it was found that log phase cells are most susceptible to a wave length of 254 mμ. At this wave length, DNA synthesis stops immediately, while that of RNA continues for one generation thereafter. These results are in harmony with those of Kanazir & Errera cited earlier (122). In other studies (114), heat treatment was used as a means of synchronizing asexual reproduction of the ciliate *Tetrahymena pyriformis* strain W. During heat treatment nucleic acids and their precursors accumulate in the organisms; in particular, the amount of DNA is doubled. This doubled DNA is partitioned between the daughters resulting from the first synchronous division following treatment. The reaction to the ultraviolet doses employed was delay in division. This delay was least great after heat treatment and greatest after the first synchronous division. One possible conclusion, that of the authors, is that ultraviolet sensitivity of these organisms, at least with respect to asexual reproduction, is inversely related to their DNA content and directly related to the amount of DNA which has to be synthesized before division. An alternative explanation, consistent with the findings, is that the ultraviolet sensitivity is inversely related to the DNA precursor content of the organisms. Romig, Socolofsky & Wyss (185) have performed somewhat similar experiments with temperature-induced, synchronously-dividing cultures of *E. coli*. As in the previously mentioned studies, these workers found that the period of greatest sensitivity to ultraviolet light appears to follow division, and the period of greatest resistance precedes division. While examining reversion to prototrophy in a histidineless strain and mutation to streptomycin resistance in another strain, they observed, on the contrary, that the number of induced mutants among the survivors was greatest in cells exposed to ultraviolet just prior to division. It would be interesting to know in this case how the internal concentration of nucleic acid precursors is correlated to cell division. According to a hypothesis discussed below, it is the precursor concentration rather than the preformed nucleic acid concentration that is important in the mutagenic effect of radiation.

The results achieved by Witkin described above are closely paralleled by those of two other groups of workers, Kimball and his colleagues working with x-ray-induced mutations in *Paramecium aurelia* (124), and Haas &

Doudney working with mutations in *E. coli* B giving aberrant color responses on fermentation-detecting (EMB, Eosin-Methylene Blue) medium (91). The work with *Paramecium* resulted in conclusions fairly similar to those of Witkin. Exposure of irradiated animals to streptomycin, which severely reduces cell division, results in a decreased number of x-ray-induced recessive lethal and deleterious mutations. Possible selective effects have been excluded by experimental test, leaving as the sole conclusion the possibility that streptomycin modifies in some way the process of mutation itself. The streptomycin effect, moreover, can be produced only in animals irradiated during the first half of the interdivision interval and only when the treatment is started within less than one hour after irradiation. On the basis of these results, Kimball *et al.* hypothesized that at least a part of the mutational process is not completed until late interphase, which is perhaps equivalent to the time of chromosomal (DNA?) replication. Until this part of the mutational process is completed, it is subject to spontaneous reversal. By supposing that more time be allowed for this reversal to occur or that the rate of the recovery process be increased, directly or indirectly, one can explain why agents capable of retarding growth reduce the yield of mutations.

Haas & Doudney (91) confirmed Witkin's finding that the yield of ultraviolet-induced mutations is dependent upon postirradiation protein synthesis. However, they also demonstrated conclusively an interesting mutation enhancement caused by preirradiation incubation in the presence of a combination of purines and pyrimidines. Cultures of *E. coli* B synchronized in division were used to compare the physiological and cytological state of the cells with the observed increase in frequency of fermentative mutants brought about by the presence of these bases in the medium. A combination of these bases was found to be necessary to enhance the mutant yield from irradiation, rather than any single base alone. Furthermore, the combination had to be supplied before irradiation, since increased purine-pyrimidine concentration was without effect after the first nuclear division. The maximum increase in the response of the purine-pyrimidine-incubated cells to ultraviolet light occurs prior to and during the first nuclear division. Upon the completion of nuclear reproduction, no additional mutational increase is observed until cell division has been completed. The frequency of mutations dependent on the purine-pyrimidine concentration increases further after cell division. These results indicate a close correlation between maximum increase in mutation frequency and periods of maximal nucleic acid synthesis.

An important fact was established by these experiments. There is no correlation between nucleic acid content of the cell and response to ultraviolet light. Cultures incubated in the basal growth medium or in the medium supplemented with purines and pyrimidines showed comparable RNA and DNA contents but yielded quite different frequencies of mutants. The findings of Haas & Doudney are consistent with the hypothesis that the

amount of nucleic acid precursors in the cell during exposure to irradiation is the important factor in mutation, rather than the amount of nucleic acid already formed prior to irradiation. It is highly suggestive that purine and pyrimidine-bearing nucleic acid precursors, possibly nucleotides, are activated by irradiation, and that utilization of these activated precursors in the synthesis of new nucleic acid molecules results in stable mutant facsimiles of the parental type. Incorporation of these "mutated" precursors is presumably dependent upon postirradiation protein synthesis, which could be accounted for if nucleic acid synthesis in some way required concomitant synthesis of protein. A recent report by Haas & Doudney (92) indicates that the increased mutation frequency caused by preincubation with purines and pyrimidines is related to RNA synthesis rather than to DNA synthesis, since neither the purines, pyrimidines, nor the deoxyribotides of DNA show any effect.

Perhaps it is yet too early to formulate a fairly detailed model of induced mutations. It seems likely, however, that disturbance of nucleic acid synthesis is related to the mutational process, and that the formation of mutant genetic material is dependent upon concomitant protein synthesis. Two reports of induced mutation caused, apparently, by disturbance of normal DNA synthesis are very interesting in this regard. Litman & Pardee (151) have found that the incorporation of 5-bromouracil into the DNA of bacteriophage T2 resulted in a greatly increased production of plaque-type mutants. [See also Zamenhof *et al.* (230, 233) for discussion of incorporation of 5-bromouracil into the DNA of *E. coli.*] The compound 5-bromouracil replaces the pyrimidine thymine in the bacteriophage DNA when the virus particles are produced in the simultaneous presence of this analogue and sulfanilamide. Selective survival of the mutant types under these conditions was ruled out. Although no report has yet been made of the relation between the amount of 5-bromouracil incorporated and the yield of mutants, it is unlikely that the incorporation of the analogue per se is the cause of the increased mutations. A large fraction of the phage containing 5-bromouracil are noninfectious, and some of the infectious phages containing 5-bromouracil are not of the mutant type. The plaque-morphology mutants induced by the analogue are stable, however, and do not need 5-bromouracil in their DNA structure in order to reproduce their type. At the present time, little can be said about the mechanism of this mutation other than that it involves, very likely, an interference of DNA metabolism. Using a histidineless (H−) derivative of the thymineless strain of Cohen & Barner, Coughlin & Adelberg (42) discovered that the unbalanced growth which occurs in the *E. coli* 15 T− bacteria in the absence of thymine results in a greatly decreased mutation from H− to H+. By the use of reconstruction experiments in which T− H+ cells containing an additional marker (norleucine sensitivity, $N^S$) were added to a population of T− H− (and norleucine resistant, $N^R$) cells, it could be shown that the unbalanced growth resulting from thymine starvation was not conferring selective advantage

upon the H+ type of cell. The finding that increased mutation and specific inhibition of DNA synthesis proceed simultaneously, lends further evidence to the theory of mutation as a consequence (or by-product) of deranged DNA synthesis.

The problem of spontaneous mutations is particularly vexing because by the mere use of that term, one appears to be confessing to an ignorance of their origin. If one assumes that the mechanism of spontaneous mutation is not essentially different from that operating in induced mutation, we need only to extrapolate from the conditions under which the mutation rate may be increased by mutagens to the probable state of affairs which may exist in the case of the cell undergoing spontaneous mutation. Results such as have been summarized above suggest that active metabolism associated with cell reproduction is essential to mutation, and that the spontaneous mutation is perhaps an error in DNA replication which occurs occasionally in the reproductive metabolism of the cell. Using the continuous culturing device known as the chemostat [for a recent review, see (166)], Novick & Szilard (167) have been able to show, in apparent contradiction to the "replication error" hypothesis of mutation, that the number of mutations to phage T5 resistance in the B strain of *E. coli* was the same per hour of growth regardless of the growth rate (generation times varying from 2 to 12 hr.). Fox (68) and Moser (161) have found, however, that when the generation time becomes very short as occurs in complex media, the rate of mutation per hour does depend on the rate of multiplication. Ryan has addressed himself to this important problem: Do mutations require replication of genetic material for their completion? As a first approximation, the question was reformulated in terms of whether or not genotypic changes occur in bacteria in the absence of cell division. Several reports have been made in the past of spontaneous mutation in nondividing bacteria (52, 186, 219a.) Ryan (187) has recently summarized the results of his own investigations on mutations from the histidineless (H−) to the histidine-independent (H+) condition which occurs in histidine-exhausted, stationary phase cultures of *E. coli* strain 15. On the basis of the following experimental results, it was concluded that the only hypothesis not excluded is that these mutations occur in the absence of cell division. In the first place, it could be shown that the H+ mutations appearing in the stationary phase did not result from mutations occurring during the period of active growth of the culture, and which were slow growing or were only detectable as such during the stationary phase. The delay in phenotypic expression of H+ is very short and not greater than one generation. The mutants appear at a constant rate in the stationary phase, and they have a maximal growth rate from their inception without a long delay in the onset of their division. Finally, it could be demonstrated that no cryptic growth was occurring in the stationary phase in a way such that lysed cells were constantly being replaced by fresh cells utilizing materials in the lysates. Reconstruction experiments in which H− Lac+ were mixed with H− Lac− cells reveal that no

change in proportion occurred in the presence of lactose (but with continued histidine exhaustion). Furthermore, no more death occurred in the stationary phase in the presence of penicillin than in its absence, except when histidine was added in amounts equal to those hypothetically released into the medium during cell turnover. Finally, even in the presence of penicillin, where growing cells could not survive, the H− to H+ mutation occurred at a maximal rate.

Despite these findings, which validate the argument that mutation does not require cell reproduction, it is possible that mutations require macromolecular synthesis, which may nevertheless occur in the absence of division. Ryan (187), indeed, observed that the rate at which mutations occurred was a direct function of the intensity of metabolism. If an exogenous carbon source were not being metabolized in the histidine-depleted cultures, mutations could not be detected when utilizable sugar was added later. Moreover, if a carbon source was present to which the H− bacteria were fully adapted, they mutated at a reduced rate. The more readily utilizable the carbon source, the higher was the rate of mutation. Indeed, mutations occurred most rapidly during growth of the culture. During the stationary phase, the cells enlarge somewhat although the number of nuclei does not increase. No modification of the mutation rate was observed in preliminary experiments in which protein synthesis was restricted by chloramphenicol or DNA synthesis by adenine starvation (in adenine-requiring strains).

Ryan & Kiritani (188) have studied the temperature coefficient of the mutation from H− to H+, by measurement of the mutation rate during growth and in the stationary phase at different temperatures. During growth, the rate of mutation per mutable unit per hour was found to have the same temperature coefficient as the rate of growth. It could be concluded, therefore, that the rate of mutation per mutable unit per generation is independent of temperature. Within experimental error, the temperature coefficient of the mutation rate per unit per hour in the stationary phase was the same as that during growth. One possible conclusion is that the same metabolic activities underlying spontaneous mutation during growth also occur in the stationary phase.

Investigations have continued of radiation and chemical mutagenesis, and of reversibility by postirradiation treatment. Westergaard (222) has admirably reviewed chemical mutagenesis in relation to the concept of the gene. Certain important generalizations are beginning to emerge from the considerable experimental results which have been obtained to date. One of these deals with the relative mutability and mutagen specificity of different gene loci and of different allelic states of the same locus. Glover (78), in a comparison of ultraviolet and x-irradiation and of several chemical mutagens on 24 different mutant strains of *E. coli* B and B/r, added further evidence to the belief (45) that mutagens are relatively specific in their ef-

fectiveness on different gene loci. The residual genotype was also shown to have an important influence on the mutability of a given type of mutation by a specific mutagen. Hartman (96) arrived at similar conclusions on the basis of studies of a number of mutagenic agents on 8 galactose-nonfermenting (Gal−) and 15 serine-requiring (se−) strains of *S. typhimurium*. Transduction experiments had revealed that the 8 Gal− strains represented nonidentical allelic mutants of the same gene locus, and that all the 15 se− strains represented nonidentical allelic mutants at a different locus, with the exception of one of the se− strains which appeared to represent still another locus. A positive correlation was observed between spontaneous and induced mutability. The results suggested, furthermore, that stability and specificity with regard to a mutagen were properties, not of the gene locus, but of the particular allelic state involved. Durham & Wyss (51) have also observed that ultraviolet light and manganous chloride have different activity for inducing mutations to streptomycin resistance and to threonine independence in a streptomycin-sensitive, threonine-requiring strain of *E. coli* K 12 and in a similar strain of *E. coli* B. Manganous chloride induced more reversions to threonine independence than to streptomycin resistance in both strains, but ultraviolet light appeared to have no differential effect. Woodward (228) devised a method for measuring mutation frequency in *Neurospora* conidia which takes into consideration a possible differential viability of mutant and wild-type spores. In examining two different mutations, one concerned with glutamic acid and the other with citrulline independence, he found that the spontaneous mutation rate of the former was about 160 times greater than the latter. The mutation rates at both loci increased with an increasing x-ray dose.

In most of the investigations described in the previous paragraph, it is possible to raise the objection that mutations or reversions were not being scored at a specific locus, and that suppressor mutations at nonrelated loci were part of the total class of mutations produced by the mutagen in question. By use of the so-called back-mutation test in *Neurospora* (see 222), conventional genetic tests can determine to what extent reversions, say from adenine-dependence to adenine-independence, are due to back-mutations at (or very close to) the locus of the original mutation. By the use of this test both Giles (76) and the Westergaard group (120) have reported earlier that different allelic mutants appear to have different spontaneous and radiation-induced back-mutation patterns. In an extension of this method, Kølmark (127) has examined the effect of a homologous series of esters of sulfate, sulfite, phosphate, phosphite, and iodide on back-mutation at two unlinked loci of a double (adenineless and inositolless) mutant of *Neurospora*. The use of this double mutant allows study of back-mutation at two different loci against an otherwise similar genetic and physiological background. Only the methyl, ethyl, and propyl esters were found to be mutagenic. Every active mutagen produced more reversions in the adenineless

locus than in the inositolless locus, but the proportion between the two reversion frequencies (at optimal reversion conditions) appeared to be a specific property of the mutagen.

An important discovery has recently been made by Giles and his colleagues (77). Several mutants of independent origin have been obtained at a single locus (ad-4) which appears to control the specificity of the enzyme adenylosuccinase (which catalyzes the splitting of AMP-succinate in the terminal step of adenine synthesis). The mutants are incapable of producing this enzyme. Back-mutations at this locus restore the wild-type ability to make adenylosuccinase. The enzyme level of some of the back-mutants appears, however, to be below that of wild-type and, in some instances, the enzyme is less stable. This finding raises the important question of the definability of the "wild-type" allele. Since it has been shown that restoration of an apparent wild-type phenotype by back-mutation at the original locus of mutation does not necessarily mean the restoration of a unique "wild-type" gene configuration and that, on the contrary, several "wild-like" allelic structures can be supposed to exist, a certain difficulty arises in interpreting the results of studies in mutagenesis. How heterogeneous is the class of so-called "back-mutations" induced by a given mutagen? How do loci differ in respect to the differentiability of their reversions from the original "wild-type" state? How can these factors be taken into account in measuring the relative specificity of mutagens?

With respect to the reversibility of induced mutations by postmutagen treatment, several new reports have been made. Jagger & Latarjet (119) found that the action spectrum for the photoreversibility of ultraviolet-irradiated *E. coli* B/r is identical with that for phage T2 in B/r. Photoreactivation is nearly constant between 3200 and 4200 A with a narrow valley at 3650 A and a valley of less importance at 3240 A. The nature of the chromophore was not identified on the basis of the action spectrum. However, Latarjet & Beljanski (129) furnish evidence against Kelner's hypothesis (123) that porphyrins are responsible for photoreactivation. Two mutants of *E. coli,* incapable of synthesizing protoporphyrin or its porphyrin precursors, were found to be as photoreactivable as the wild-type. Fenn *et al.* (61) have reported a mutagenic effect of high oxygen pressures in reversions of a streptomycin-dependent strain of *E. coli* to the antibiotic-independent condition. Consistent with the assumption of oxygen as a mutagen, or as a synergistic agent in mutagenesis, are the findings of Marcovich (157) which indicate that the protection provided to bacteria by cysteamine against radiation arises from its ability to deprive the medium of oxygen.

An interesting new way of attacking the problem of mutagenesis is through the study of mutagens on transforming DNA preparations. No reports, however, have been made of any production of mutant DNA in this way. The mutagens seem to inactivate exclusively, rather than to confer any new genetic property on the "naked" DNA. Goodgal, Rupert & Herriott (81) have, nevertheless, described the photoreactivation of ultraviolet-

irradiated, transforming DNA from *Hemophilus influenzae*. It is interesting, in this regard, that an extract obtained from *E. coli* (and not obtainable from the notoriously porphyrin-poor *Pneumococcus* or *Hemophilus*) was found to be necessary in the medium of the irradiated DNA in order for photoreactivation to occur. Since the studies of physical and chemical agents on the inactivation of transforming DNA are, at the present time, more interesting in what information they can furnish about the mechanism of transformation itself, they will be reviewed in the next section.

### Replication

If DNA is the material substance of which the genetic apparatus of cells is composed, then it is not only important to understand how it mutates, but how it replicates as well. For it is by the replication of DNA that a mutant configuration is transmitted to the successive generations of cells and, indeed, as it has been suggested above, the very nature of the replication process may set the conditions of the mutational event. An excellent critical discussion of our present knowledge of DNA replication was provided recently by Delbrück & Stent (44). They refer, for the most part, to experimental work with the bacteriophages, the presumable material of choice for this problem. In Delbrück and Stent's classification, assuming a duplex structure of DNA, the ways in which parental genetic material may conceivably be distributed between the daughter DNA duplexes are three: (*a*) conservative, in which one entire daughter duplex is fabricated from nonparental material and the other daughter duplex is the parental structure; (*b*) semiconservative, in which each daughter duplex contains one chain that is old (parental) and one that is new; (*c*) dispersive, in which the distribution of parental material is uniform through the four chains of the two daughter duplexes. By the use of cells in which the DNA is labeled with a radioactive marker, several experiments have been performed to discriminate between these possibilities of DNA replication. Plaut & Mazia (174) allowed chromosomes of the plant *Crepis capillaris* to incorporate $C^{14}$-labeled thymidine for a time sufficient for cells to go from early interphase to telophase. The radioactivity contained in the six chromosomes of each of the two sister nuclei in a number of telophase figures was measured by autoradiographic techniques. An unequal distribution of parental label was found between pairs of chromosomes. The frequency distribution of the magnitude of these inequalities seemed to indicate *de novo* synthesis of the six daughter chromosomes, followed by such random alignment of the daughter and parental chromosomes on the metaphase plate that there is a random migration to the poles of nonlabeled mother chromosomes and labeled daughter chromosomes. Dispersive and semiconservative replications seemed to be ruled out in this case. Taylor, Woods & Hughes (214), however, arrived at other conclusions in the bean plant *Vicia faba* by means of a somewhat different technique. The bean roots were placed in a mineral medium from which they incorporated tritium-labeled thymidine for 8 hr., or approximately one-

third of the division cycle. The roots were then transferred to a nonradioactive medium containing colchicine, which is known to arrest anaphase movement but does not block chromosomal replication. After appropriate intervals (to allow for one or more chromosomal duplications) roots were fixed, stained by the Feulgen method, squashed on slides, and autoradiographs prepared. The tritium label was chosen because its decay particle has a very short track, of little more than one micron in photographic emulsions, and is thus ideal for identification of the label in individual chromosomes. The autoradiographs showed that both daughter chromosomes resulting from the duplication in the presence of labeled thymidine are equally and uniformly labeled. The label appeared in only one of each of the two daughter chromosomes after duplication in the absence of the label. This evidence is consistent with the postulated duplex structure of the chromosome and with a semiconservative model of replication. Levinthal (147, 148) has obtained results with replication of coli-phage DNA that are, in most respects, similar to those of Taylor *et al.* In these studies the label used was $P^{32}$ and the radioactivity of the DNA "molecules" in virus particles of the first and ensuing generations of labeled phage was measured by autoradiography. Levinthal found that the phage particle contains DNA units of different size. One piece of DNA contains about 4 per cent of the total DNA, and it is to this piece that the genetic markers of the phage are associated. This large piece replicates to give two particles, each of which contains one-half its atoms. No further distribution of the original atoms of the large piece takes place upon further replication. So far as the small pieces are concerned, not much is known except that none appears to contain more than 8 per cent of the total phage DNA.

Fuerst & Stent (72) have shown that the decay of $P^{32}$ atoms incorporated in the DNA or, as is assumed, in the "nuclei" of *E. coli* destroys the ability of the bacteria to produce colonies, the rate of inactivation being proportional to the specific $P^{32}$ content of the DNA. They compared the reduction in rate of $P^{32}$ inactivation in a culture, starting with initially unlabeled cells grown in the presence of the label, with that observed in a culture originating from labeled cells grown in a nonradioactive medium. It was found that more than 99.9 per cent of the cells from an initially unlabeled culture which had incorporated $P^{32}$ for only slightly more than one generation was subject to $P^{32}$-inactivation in a way indicating that replication had distributed the DNA atoms of each bacterial "nucleus" equally between its daughters. When a $P^{32}$-labeled culture was allowed to grow for slightly over a generation in unlabeled medium, the rate of inactivation was decreased as if an equal partition of the radioactive phosphorus atoms had taken place between the daughter nuclei. Upon subsequent divisions in an unlabeled medium, however, a minority of cells appeared which were less sensitive to $P^{32}$ inactivation than if equal partitioning of the original phosphorus atoms had continued. Nevertheless, after four generations no completely $P^{32}$-insensitive bacteria had yet appeared. These results are compatible with the

semiconservative model of replication, with the proviso that the continuation of $P^{32}$-sensitive bacteria after the fourth generation comes from random segregation of different "chromosomes" or pieces of DNA genetic material.

Forro (67) has followed the distribution of $P^{32}$ among progeny of a single labeled cell of *Micrococcus cryophilus* through the eighth generation by using micromanipulative and autoradiographic methods. The results indicate that the DNA of this bacterium cannot be organized into less than 25 stable units whose prosphorus integrity is maintained on cell division. Indeed, there cannot be one such unit with more than 10 per cent of the cell's DNA. Unfortunately, this information cannot bear directly on the question of the mechanism of replication of these units. Painter, Forro & Hughes (171) have studied the distribution of tritium-labeled thymidine during multiplication of the thymineless mutant of *E. coli* strain 15. The bacteria were grown for six generations in a medium containing the labeled nucleoside, after which it appears that no less than 80 per cent of the label is in DNA thymidine. Two types of experiments were performed. In the first, the cells were washed and transferred to a medium containing unlabeled thymidine. Every time a doubling of the population occurred, as measured by direct cell count, smears were made, autoradiographs taken of them immediately, and the bacteria stained some time afterward. In the second type of experiment, the labeled cells were isolated by means of a micromanipulator onto nutrient agar containing unlabeled thymidine. Clones were derived from these isolates, growth was stopped at a given generation by drying the agar medium, and autoradiographs were made after fixation. Both types of experiments showed clearly that there was no simple dilution of the label; rather, there was a very heterogeneous distribution in the progeny of the labeled bacteria. Not at the end of the first, but by the third generation, some cells without label were observed. Others still had a great deal of label. Occasionally, a pair of cells which had not yet separated were seen to have a very heavy label in one cell and none in the other. The second type of experiment revealed one important fact, that each labeled cell does not give rise to a unique number of labeled cells dispersed among other unlabeled ones. Rather, the distribution of label varies greatly from one clone to another, but in some clones the distribution appears to be random. Although these results were preliminary, and the frequencies of these patterns of label distribution need to be further investigated, the general conclusion seems to be warranted that in *E. coli* relatively large amounts of parental DNA can remain associated in transmission to daughter cells. That is to say, even if the number of "chromosomes" per bacterium is large, or if the "chromosomes" fragment into numerous smaller pieces during transmission, the evidence is now good that the dispersive model of replication does not hold for bacterial DNA and that, in view of the absence of unlabeled cells after the first division in the above experiment, the conservative model seems to be excluded as well.

The semi-conservative model for replication of bacterial DNA seems to be supported as well by exciting new results of Meselson & Stahl (157a), utilizing the method for resolving macromolecules by equilibrium sedimentation in a density gradient (157b). By this method, DNA containing $N^{15}$ can be separated from normal DNA (containing $N^{14}$). Fully $N^{15}$-substituted, exponentially growing cultures of *E. coli* were transferred to $N^{14}$ medium. When the population doubled, all DNA molecules were found to be half-labeled with $N^{15}$. In all ensuing generations, the number of such half-labeled molecules remained constant.

The use of bacterial cultures in which cell multiplication is synchronized may prove to be of great value in genetic experiments (as exemplified above). The subject of synchronization has been reviewed recently by Campbell (28). There is some evidence to suggest that the induction of synchronization by temperature shifts arises from a change in rate of DNA synthesis. Lark & Maaloe (128), for example, found that in raising the temperature of a culture of *Salmonella typhimurium* from 25° to 37°C., the synthesis of DNA seems to be triggered, which leads at once to nuclear reproduction. Scott & Chu (195) have investigated two ways of inducing synchronous division in *E. coli* B. These are by chilling and rewarming a growing culture, and by adding glucose to a culture which has been incubated for at least 4 hr. after depletion of glucose in the medium. During the period of depletion the ratio of RNA to DNA fell, and it rose again after addition of glucose. Falcone & Szybalski (60) have measured several biochemical properties in cultures of *Bacillus megaterium* undergoing 1 hr. of chilling after previous active multiplication. Cell division stops completely within the first 30 min. of the chilling period. Cell multiplication is synchronized upon subsequent incubation at 34°C. The halt in cell multiplication during the chilling period could not, however, be correlated with a similar arrest of synthesis of any of the measured components. Increase in dry weight and turbidity of the culture are least affected. The syntheses of DNA, RNA, and protein are inhibited in increasing order. Falcone and Szybalski suggest that synchronization results from a period of unbalanced growth which, by favoring DNA synthesis, may bring all of the nuclei into the same phase.

## TRANSFER OF GENETIC MATERIAL

To date, several well-defined modes of transferring genetic material from one bacterial cell to another have been described. These modes of transfer have been reviewed recently by Hartman (95) and by Lederberg (134, 138). The modes of transfer may be distinguished operationally as follows: (*a*) "Transformation" refers to the transfer into recipient bacteria of genetic determinants from a cell-free, phage-free, deoxyribonuclease-sensitive DNA preparation. The transfer is detected because the transformed cells correspond in character to one or more heritable attributes of the "donor" cells from which the transforming preparation was extracted. *(b)* "Transduction"

refers to the nonobligatory transfer by bacteriophage into another bacterium, of a fragment of the genome of the bacterium it has previously infected. In the extracellular form the material transferred is resistant to deoxyribonuclease, presumably because it is protected by the same phage coat as protects the phage DNA itself. In order to detect the newly acquired character in the progeny of the recipient bacteria, the phage vector must be temperate (i.e., capable of lysogenizing), but transfer of the determinant and lysogenization need not occur simultaneously. (*c*) A special case of phage-mediated genetic transfer is "lysogenic conversion." Here the establishment of a prophage confers obligatorily some new genetic property upon the cells comprising the lysogenic clone. (*d*) "Conjugation" requires cell-to-cell contact, and is characterized usually by unidirectional transfer of several different determinants at once. (*e*) "Heterokaryosis" refers to the anastomosis of protoplasm of genetically different cells, so that nuclei of different genetic constitution are present in a common cytoplasm. Finally (*f*), recent experiments on "infection of protoplasts" indicate a possible mode of genetic transfer which has been relatively unexplored heretofore. Although much of the work is still in a preliminary stage, it will be discussed because of its importance for future research.

## Transformation

The bacterial species in which transformations have been reported are many, although the phenomenon is being studied in a systematic manner in only a few. This is certainly attributable, at least in part, to the complexity of the conditions essential for reproducible transformations, which has been studied carefully only in *Pneumococcus* and *Hemophilus*. Yet, it is important to know whether transformation is limited as a mechanism of genetic transfer among species of bacteria, for it represents a powerful means of increasing genetic variability through recombination in strains lacking bacteriophages or a means of establishing conjugation bridges between cells. Corey & Starr (39, 40, 41) have recently reported reproducible transformations involving capsule synthesis and streptomycin resistance in the Gram-negative plant pathogen, *Xanthomonas phaseoli*. Some of the features of the transformation system in *Hemophilus* and *Streptococcus-Pneumococcus* have been observed in transformations of *Xanthomonas*, such as phenomic lag in the appearance of streptomycin-resistant transformants and competition between DNA "molecules" in infection of the recipient strain. Brown *et al.* (23) presented evidence in 1955 of an interesting kind of transformation which was best explained as being caused by a bacteriophage serving as a vector of transfer for transforming DNA fortuitously but firmly bound to the exterior of the virus particles. Nonmotile strains of *B. anthracis* were presumably transformed by a cell-free phage preparation obtained from a motile variant. The activity of the preparation was lost by treatment with deoxyribonuclease, but it could not be separated from the bacteriophage by centrifugation. Unfortunately, a recent report (207) casts

doubt on this finding, and one must wait further reports of the reproducibility of the original phenomenon.

In recent years, several reports have been made of transformations between species which are regarded, in most schemes of bacterial taxonomy, to be different species. Schaeffer (190) described the transformation to streptomycin resistance of *H. influenzae* cultures treated with the DNA from a resistant mutant either of the same strain or of *H. parainfluenzae*. The manner in which the recipient strain becomes competent (see below) and in which the infected cells express their newly acquired character, appears to be the same for intraspecific and interspecific transformations. However, the frequency of the interspecific transformation is about $10^4$ times lower than that of the intraspecific transformation. Similar findings were made by Leidy, Hahn & Alexander (139) who extended the investigation to several different species of *Hemophilus*. They found that a given strain may serve either as the recipient of DNA from some other species or as a donor of DNA to that species. In a given recipient strain, the frequency of the interspecific transformation was always lower than that of an intraspecific transformation and, in general, the ratio of the frequency of interspecific transformations to that of intraspecific transformations correlated well with the degree of kinship between donor and recipient cells as suggested by current schemes of bacteriological classification. Reciprocal transformations of streptomycin resistance have also been observed by Bracco *et al.* (19) between pneumococci and two strains of viridans streptococci. Furthermore, a strain of *Streptococcus salivarius,* which was not transformable by its own or heterologous preparations, nevertheless yielded DNA extracts which were capable of transforming pneumococci and two strains of *Streptococcus viridans*. Another interesting observation was that resistance to optochin, a characteristic of most streptococci, could be transferred to the naturally susceptible pneumococcus via DNA extracts.

In two previous reports (110, 213) it had been shown that the transformation reaction does not consist of the simple addition of a genetic determinant to the host's genome, but rather of the replacement of a determinant already present in the genome by a homologue present in the transforming preparation. Thus, reciprocal transformations between the nonfilamentous and the filamentous type of growth were known (213), as well as reciprocal transformations between mannitol-oxidation and mannitol-nonoxidation and between streptomycin resistance and streptomycin sensitivity (110). By screening for cells transformed for the capsular character among the class of cells also transformed for the streptomycin-resistant character, Ravin (181) has now shown that normal and deficient capsular synthesis are also reciprocally transferable. He also observed a quantitative diminution in the transformability of a recipient strain as its production of capsular polysaccharide increased.

Hotchkiss (108) has recently reviewed the evidence for genetic heterogeneity in the transforming units present in the DNA preparation from a

given strain of donor bacteria. In general, when donor and recipient cells differ by more than one genetic attribute, several classes of transformed recipients are obtained corresponding to single and multiple "gene" transfers. The frequency of multiply-transformed bacteria is low as compared to that of the singly-transformed bacteria, and it is roughly what one would expect if transforming units bearing different genetic information were present in a DNA extract and were incorporated independently of each other. Occasionally, two genetic determinants or "markers" are not transferred in an independent fashion (110). Goodgal & Herriott (79), thus, have furnished evidence that the DNA from a doubly mutant strain of *Hemophilus influenzae,* resistant to both streptomycin and cathomycin, produces 100 times more doubly-resistant transformants in a sensitive recipient strain than does a comparable mixture of unlinked factors. The linkage between the determinants was shown to be independent of the concentration of transforming DNA employed, even at nonsaturating concentrations. The evidence for linkage indicates that the marker being transferred in a given type of transformation is smaller than the average DNA particle in the transforming extract. The distribution of the markers on the various DNA particles becomes an important question. Are DNA particles or "molecules" uniform in regard to their composition of genetic markers? If so, then the host bacterium must fragment the infecting particle such that usually only single markers are incorporated, and a greater than random incorporation of certain double markers may be due to their close proximity on the DNA particle. Alternatively, are DNA particles heterogeneous in their composition? If so, this condition could arise from the dispersion of these markers over a large number of genetic particles or chromosomes in the bacterial cell, or it could come from the process of extracting and purifying the transforming DNA in which fragmentation of a few large "chromosomes" into many smaller particles may be unavoidable (or nearly so). One hope of answering these questions lies in physical studies of transforming DNA, which will be discussed below.

Several new studies concern the number of loci and allelic states affecting some specific character of the bacterium. Hashimoto (97) has confirmed previous findings of Hotchkiss (103) which stated that resistance to high levels of streptomycin, acquired in a series of steps and involving presumably independent mutations, is not transferred via transforming extracts to sensitive recipient cells in a single step. The upper level of resistance can be acquired only after successive treatments with the same extract. High-level resistance acquired by a strain in a single mutational step can be transferred, on the other hand, with high efficiency through its DNA extract. These results are consistent with the view that many unlinked loci are involved in streptomycin resistance, and that a single locus may mutate to confer as much resistance upon a cell as a group of "resistant" loci. Schaeffer (191) has isolated four strains of pneumococci which are resistant to different levels of streptomycin. Three of these (r1, r2, and r3) appear

to represent allelic mutants. The fourth (r4), appears to be a multiple mutant. Sensitive cells transformed by the DNA from r4 are resistant to a lower level of streptomycin than is the donor strain itself. To raise the level of their resistance requires a second round of treatment with the same DNA. The DNA's from r1, r2, and r3 are specific; that is, they transform to levels of resistance characteristic of the corresponding donor strains (where level of r3 > r2 > r1). While a mixture of two of these DNA's produces two classes of transformants in regard to resistance level, a DNA from a transformant obtained in two steps (sensitive + DNA r1 → r1; r1 + DNA r3 → r3) produces transformants resistant only to the level that the donor is capable of withstanding (there is, in other words, no trace of the r1 agent in DNA r3). The DNA's from r1, r2, and r3 behave, quantitatively, quite differently on the sensitive recipient strain. The frequency of induced transformants apparently rises linearly with the increasing dose of a given DNA, but reaches a plateau characteristic of the DNA which is being used. Thus, the maximum frequency of transformants depended upon the marker involved (frequency of r3 > r2 > r1). The effects of possible inhibitors in the transforming preparation were ruled out by the results of mixture experiments. To explain the differences in transformation frequency with DNA r1, DNA r2, and DNA r3, Schaeffer proposed either: (*a*) different mutations can occur in the structure of a locus, and that, possibly, the greater the length of the locus affected, the less chance for incorporation; or (*b*) each allelic state confers a different probability for the physiological accomplishment of resistance possible with that marker. MacLeod & Krauss (156) have shown that different loci cooperate in the control of capsule production in the Type VIII strain of pneumococcus.

The genetic conversion of cells in a growing culture by DNA determinants present in the medium is now known to occur in a series of discrete steps. First, the recipient cells must be physiologically competent to react with the transforming DNA. Thomas (215), in an earlier work, studied the kinetics of the acquisition and the decay of competence in a growing culture. Suffice it to say that competence is gained and lost cyclically, and the rate of appearance and decay of this physiological state is extremely rapid, so that a culture passes through a peak of competence within a fraction of the generation time. Hotchkiss (106) achieved some measure of success in synchronizing the gain of competence in a synchronously dividing culture induced by temperature shifts. Fox & Hotchkiss (70) have now reported some interesting results which indicate that the development of competence consists essentially in the appearance of bacterial adsorbing sites with which transforming units of DNA combine reversibly. The dissociable complex (DNA-bacterial adsorbing site) may then undergo an irreversible change (fixation or incorporation of the DNA) resulting in a transformant (a cell which is on the irreversible road of producing some progeny of transformed phenotype). Populations of competent pneumococci were frozen in 10 per cent glycerol and stored at −20°C. Such cells remained viable for at least

18 months and retained their competence for as long as three months. The frozen populations were allowed to thaw to a chosen temperature, DNA was added, aliquots were removed at various times when the reaction was terminated by DNase; the aliquots were incubated at 37°C. for a period of time sufficient to allow for expression of the transformed character. It was found that, after a short induction period, the number of transformants increased linearly with time of exposure to DNA. The linear increase continued for over an hour, during which time no cell multiplication occurred. The slope of the linear rise was sensitive to the temperature at which cells contacted the DNA, and the optimal temperature was between 30° and 34°C. If the slope (or rate of initiation of transformation) was plotted as a function of DNA concentration when the concentration of competent cells was held constant, or as a function of the concentration of competent cells when the DNA concentration was held constant, Fox & Hotchkiss found that the rate was directly proportional to the DNA concentration or cell density except at very high concentrations when saturation occurs. These results are explicable on the basis of a conventional model of enzyme-substrate complex formation:

$$B(s) + \text{DNA} \underset{k2}{\overset{k1}{\rightleftharpoons}} B(s) \cdot \text{DNA complex} \overset{k3}{\rightarrow} B(TR),$$

where $B(s)$ represents the number of available bacterial adsorbing sites and $B\ (TR)$ represents the number of transformants. Using the Lineweaver-Burk treatment of the data, a

$$V_{max} \text{ and } K \left[ = \frac{k2 + k3}{k1} \right]$$

can be determined. These measurements indicate that from 33 to 75 adsorbing sites are developed on the competent bacterium, and that the probability of dissociation of the DNA-adsorbing site complex is 30 times greater than the probability of irreversible incorporation of the DNA. Further studies demonstrated that not only can competence arise in the absence of cell multiplication (although expression cannot occur in the absence of growth), but it also depends upon the presence of calcium ions and of serum albumin in the medium. When competence is lost by storage at −20°C., its redevelopment depends, not only upon the presence of calcium and albumin, but also upon what appears to be protein synthesis. For the formation of adsorbing sites requires a mixture of amino acids, is facilitated by glucose as an energy source, and is completely inhibited at very low concentrations of the specific inhibitor of protein synthesis, chloramphenicol.

The stage following acquisition of the ephemeral state of competence by a bacterium involves an effective contact with the DNA particle such that at least some of its progeny will manifest the transformed phenotype. The evidence has now quite soundly established the fact that it is sufficient for a bacterium to make contact with a single transforming particle in order for

transformed progeny to appear in its clone (109). By means of transforming DNA containing a $P^{32}$ label, it has been shown by three different workers independently (69, 80, 143) that transformation is accompanied by assimilation of DNA into the host bacterium. The frequency of competent cells transformed after brief exposure (less than one-half the generation time) to transforming DNA and then washed or treated with deoxyribonuclease, is directly proportional to the amount of $P^{32}$-label incorporated. This finding is obtained regardless of the relative concentration of DNA or competent cells, or of other conditions affecting transformation frequency such as the duration of exposure to the transforming DNA or the concentration of calcium in the medium. Goodgal & Herriott (80) demonstrated in *Hemophilus* that the $P^{32}$ is incorporated into the DNA fraction specifically. Assuming a value of $1.5 \times 10^7$ for the molecular weight of DNA (see below), the number of molecules found to be taken up irreversibly for each cell transformed was 120. This number was constant over a wide range of DNA concentrations and at different levels of host competence. That no fractional uptake of transforming agents was occurring was further suggested by the fact that the cells take up their own (autologous) DNA to the same extent as they take up transforming DNA. Only two conclusions seem possible: either one in about 100 molecules of donor DNA bear the particular marker investigated (streptomycin resistance), or every molecule bears that marker and transformation has an efficiency of only $10^{-2}$. Fox (69) has demonstrated that the amount of label taken up per observed transformant varies with the state and quality of the DNA preparation. With the most effective fresh preparations, about 1.4 times the DNA content of the cell is incorporated when one cell is transformed for a given property. Older, less active preparations show more DNA incorporated per bacterium transformed, thus revealing that genetically ineffective DNA can also be taken up by the recipient cell. This finding suggests that the estimation of the number of transforming units taken up per transformant is a maximum, and may very well be less. Schaeffer (193) has arrived at a similar conclusion on the basis of results of $P^{32}$ incorporation in streptomycin-resistant transformations of *H. influenzae* by DNA of homo- and heterospecific origin. The same number of DNA molecules, as measured by $P^{32}$ uptake, is incorporated into a competent cell in either case. It follows that the relatively low frequency of interspecific transformations results from some defect in a postinfection process (perhaps in the pairing between donor and recipient DNA).

Hotchkiss (109) has furnished convincing evidence of competition between DNA molecules in transformation. Altering the ratio of the concentrations of genetically ineffective DNA (such as homologous, nontransforming DNA) and genetically effective DNA in a mixture, results in a lowered frequency of transformation at saturating concentrations only, but not at limiting concentrations. Schaeffer (192) has demonstrated in a convincing manner that the competition between DNA molecules is for pene-

tration into the bacterial wall, rather than a competition in some process following infection. $P^{32}$-labeled DNA from a streptomycin-resistant strain of *Hemophilus influenzae* was mixed with inhibiting DNA in the form of nonlabeled DNA from streptomycin-sensitive cells of the same type as were to act as recipients in the experiment. On the basis of the hypothesis of preinfection competition, one would anticipate that the frequency of transformation as well as the amount of radioactive DNA taken up would decrease with an increasing proportion of inhibiting DNA in the mixture. On the other hand, if the postinfection hypothesis of competition were correct, one would expect the transformation frequency, but not the amount of DNA taken up, to decrease. Results of the experiment were that the per cent inhibition of irreversibly bound radioactivity and the per cent inhibition of transformation fell at exactly similar rates with the increasing proportion of inhibiting DNA.

Schaeffer (194), in another experiment, utilized this means of inhibiting transformation as a measure of bacterial competence. The two hypotheses can now be contrasted: only a fraction (at best, 1 per cent) of the cells in a competent culture can react with transforming DNA, or, all can but with a low probability (say of $10^{-2}$). Discrimination between the two hypotheses can be made because they predict quite different minimal concentrations of inhibiting DNA which will produce a detectable lowering of the transformation frequency, under conditions where the "multiplicity" is low (less than 1 molecule per 10 bacteria). The results, for a series of multiplicities ranging from $10^{-3}$ to 1, are in accordance with those predicted by the hypothesis of a population in which 100 per cent of the cells are competent. Such homogeneity in competence exists, of course, only during the peak periods of competence which develop in a multiplying population. Yet, it remains to be seen whether or not there is any fundamental difference between the distribution of competence among *Hemophilus* cells, with which Schaeffer's work was done, and that found among Pneumococcus cells. With the latter, Hotchkiss (106) had found earlier that synchronization of division raised the frequency of transformation, which suggested that, at least in the absence of synchronization, competence was present in only a fraction of the cells at a given moment.

The steps following infection are now becoming clear. An infected pneumococcus gives rise to a mixed clone of transformed and untransformed progeny, indicating that the infected cell is, in actuality, heterozygous for the genetic determinant being followed (108). The proportion of transformed to untransformed progeny in the clone of a single infected bacterium appears to vary considerably from one clone to the next. One way of interpreting these results is that there is a variable lag before the determinant is incorporated into the bacterial genome. Until this genetic incorporation occurs, the determinant appears to be diluted rather than replicated and distributed in a regular manner among the progeny cells. While this lag in genetic incorporation is taking place, the presence of the genetic deter-

minant within the host bacterium may, nevertheless, act upon the phenotype. An alternative explanation for the segregation of cells of untransformed phenotype during the multiplication of the originally infected bacterium is the following: it is possible that the transforming determinant synapses with the homologous segment of the host genome and multiplies as such, or as part of a new entire genome, for a variable number of generations before this heterozygous (or hemizygous) diploid condition is reduced to the original haploid state. The former explanation corresponds to the model of abortive transduction, the latter to the model of the heterogenotic transduced clone, both of which will be described below.

As Hotchkiss has shown (105), some time elapses before the phenotypic action of the transforming agent becomes manifest. An infected bacterium which is still heterozygous for the agent in question (that is, is still giving rise to untransformed progeny) may, however, be phenotypically transformed. This is true in the case of the marker for streptomycin resistance studied by Hotchkiss (108). This finding demonstrates that, in pneumococcus, the determinant for streptomycin resistance is dominant over its allele-producing streptomycin sensitivity, and it suggests also that the heterocatalytic function of the determinant does not require prior genetic incorporation.

Hotchkiss (108) has described a number of possible models of genetic incorporation of the transforming determinant. Two can be clearly excluded by examining clones of cells treated with a transforming preparation containing two linked markers, one for mannitol oxidation, the other for streptomycin resistance. The excludable models are those which postulate that all of the infecting DNA particle is eventually utilized by the progeny of a single infected host bacterium. These models predict that some clones would contain both mannitol-oxidizing cells and streptomycin-resistant cells. However, such a finding is not made; clones are found to contain mannitol-oxidizing cells or streptomycin-resistant cells, or mannitol-oxidizing, streptomycin-resistant cells.

Several attempts are being made to determine whether or not a physicochemical heterogeneity of DNA particles underlies the genetic heterogeneity in a transforming preparation. Bendich, Pahl & Beiser (13) have obtained results with adsorption of DNA on substituted celluose (ECTEOLA) columns followed by elution with increasing concentrations of NaCl (and pH), which they interpret as revealing a physical heterogeneity of cellular DNA. The chromatographic profile of the DNA from a given source appeared to be reproducible, but it varied from one tissue to another, from one species to another, and also from one bacteriophage mutant (T6r) to another (T6r+). Pneumococcal DNA containing the streptomycin-resistant marker was chromatographed by these workers. They found marker activity in most of the fractions, but to different extents. They claimed that these differences were not related to the amount of DNA present in the fractions. However, so long as the amount of total DNA in a fraction is measured by an indicator as nonspecific as ultraviolet absorption, to what extent the

amount of marker activity is related to the amount of "native" DNA in the fraction remains an open question. The work of Fox (69), Goodgal & Herriott (80), and of Lerman & Tolmach (143) has shown that there are differences in the "quality" of various DNA preparations, and there appears to be a certain amount of genetically ineffective DNA in transforming preparations, which increases with age and other conditions of the preparation. Lerman (142) emphasizes the importance of the purity of transforming DNA preparations in estimating physical heterogeneity. In an earlier report (141), he found, using pneumococcal DNA on an adsorption column of insoluble methylated serum albumin, results essentially similar to those of Bendich *et al.* Rechromatography of a given fraction showed that it was still heterogeneous in regard to solubility in the eluent, but it seemed to be undergoing purification in the course of successive adsorptions and elutions. With present preparations, however, Lerman (142) now finds that repeated chromatography of any one fraction does no more than reproduce the original chromatogram, and hence that no purification is occurring. Indeed, the transforming activities (of the streptomycin resistance and mannitol-oxidation markers utilized in these studies) per unit weight of DNA in the various fractions were similar to each other and to that of the original preparation. Lerman attributes his latest results to the greater DNA purity of his present transforming preparations. Brown & Ephrussi-Taylor (24) were also unable to separate the streptomycin-resistance marker by chromatography of their transforming preparations. Butler, Phillips & Shooter (26) have demonstrated the considerable influence which small quantities of residual protein play in bringing about sedimentation heterogeneity in a DNA preparation. From their studies of chymotrypsin-treated fractions, they concluded that protein-DNA aggregates can contribute to high sedimentation coefficients. It was also shown that different ways of preparing DNA resulted in different amounts of residual protein. Ephrussi-Taylor (59) has obtained evidence for aggregation at very high concentrations of transforming DNA.

The outlook for the fractionation of DNA, nevertheless, is not hopeless. Brown & Martin (25) have recently reported a gentle method for removing phage DNA from its protein coat. By their method they succeeded in fractionating T2 DNA into two fractions which differed in their base ratios. Cavalieri (30) has published a preliminary communication on results obtained by the use of a glass atomizer at various pressures to cause monodispersion of DNA. The possibility for physical fractionation in this way appears promising, except for the risk of producing artefacts.

Several investigations are under way using mutagens as a tool for the analysis of DNA-transforming preparations. Tolmach & Lerman (216) have studied the effects of ultraviolet light, heat, nitrogen mustards, and DNase on $P^{32}$-labeled pneumococcal DNA. They found that the ability to transform is much more sensitive to ultraviolet irradiation than the ability to be taken up irreversibly. Hence, it seems clear that ultraviolet-inactivated deter-

minants can enter the bacterial cell. Another interesting effect of ultraviolet was observed, in that two different markers (affecting resistance to two different drugs) were not equally affected by a given dose of radiation. This differential effect was not observed, however, after treatment with nitrogen mustards, heat, or DNase. The inactivation of irradiated DNA as a function of ultraviolet exposure is complex, as has been observed by other investigators (58, 90). When recipient cells are treated with ultraviolet, it is found that a cell having lost the ability to form a colony can nevertheless take up DNA. Such an effect is not found for heat treatment.

Zamenhof and his co-workers (231, 232) have also observed the differential effect of a mutagen on two markers in a transforming preparation from *Hemophilus*. This effect was observed most readily with ultraviolet light but, although quantitatively not so marked, a similar effect was obtained with other agents such as nitrogen mustards, heat, $H^+$, and $Fe^{++}$. These workers have adopted an unfortunate term for this observation, namely, "differential stability of hereditary determinants," which presupposes the mechanism of the effect. It is possible that there is no difference in lability of the various markers, but that incorporation into the host genome may vary in difficulty for different markers in a given irradiated transforming preparation.

Guild & DeFilippes (90) have made a careful study of inactivation by ionizing radiation of transforming activity in dried pneumococcal DNA. The inactivation curve as a function of dose consists of at least two components, a rapid inactivation at low levels followed by less intense inactivation which is, nevertheless, strictly exponential at higher levels. The initial component of the curve varies with a number of conditions, such as the nature of the medium in which the DNA is dried. The second component, of shallower slope, is less difficult to reproduce, and is independent of such conditions as affect the initial component and also of ultrasonic treatment which lowers the initial transforming activity of the preparation. What has been definitely established is the fact that the two components do not represent an a priori distribution of particle sizes or sensitivities in the preparation before drying (58). This was proved by reirradiating a redried sample of DNA which had been previously irradiated with a dose that decreased its activity to a level on the second or ultimate slope of inactivation. The same two-component curve of inactivation was obtained as that of the initial dried preparation. The authors suggest that the first component of inactivation may be the destruction of DNA aggregates which are produced by drying, and which would have been redispersed if not irradiated. The second component, however, appears to represent the inactivation of that portion of the DNA molecule essential for the particular genetic marker being followed. Damages within this portion of the molecule would result in a loss of its transforming activity. The final slope of inactivation obtained for the streptomycin-resistance marker studied corresponded to a target molecular weight of $3 \times 10^8$. This figure does not necessarily represent the molecular weight

of DNA, especially if several pieces of genetic information may be carried by a given transforming unit or molecule of DNA. Indeed, Goodgal & Herriott (80) report a molecular weight for DNA of $1.5 \times 10^7$, based upon sedimentation and diffusion measurements.

Two preliminary reports have recently been made of the possible antigenicity of DNA (101, 173). These reports are, of course, of great interest and significance, and demand further study.

## Transduction

At the time of the original discovery of transduction by Zinder & Lederberg (237), it was pointed out that the bacterial determinants transferred by temperate phage are incorporated, on the whole, independently of each other, so that multiple-marker transductions are uncommon events. It is generally true that, in transductions of *Salmonella*, markers from any segment of the donor chromosome can be transferred via the temperate phage vector (235). Englesberg (55), however, has reported a gene (one affecting glucose inhibition of the utilization of Krebs cycle intermediates) which is apparently refractory to transduction. Largely owing to the work of Demerec and his group (46, 47), the complexity of the transferred determinant has been established. They showed that different sites could be recognized within a locus affecting a specific step in the synthesis of some metabolite. These sites were separable by recombination, but they tended to be transferred together by a given transducing phage. From the linkage relationships between these sites, they could be arranged in a linear sequence, presumably representing their order on the intact donor chromosome. In this way, a number of different loci could be mapped. These complex loci have been quite useful for investigations into the relation between organization and function of genetic material, and will be discussed further in this connection.

Ozeki (170) has described a type of transduction termed "abortive" which is distinguished from "stable" transduction, in the inability of the transferred fragment of the donor genome to be incorporated into the genome of the recipient bacterium. The phage-transferred gene in this case (at one of the adenineless loci of *Salmonella*) is not replicated but persists as an "extra gene" in one of the progeny in the clone of the abortively transduced bacterium. The presence of the extra gene brings about a phenotypic modification in the cell in which it is present and in a few of its progeny, and this modification allows the formation of minute colonies. The frequency of abortive transductions by phage carrying a particular gene is much higher than that of stable transductions for that gene. Stocker (209) has made a similar observation in transductions of motility in *Salmonella*. In his studies, micromanipulative isolations of progeny at various generations after infection permitted verification of the hypothesis that an "extra gene" was being transmitted in a unilinear manner within the clone of the transduced bacterium. In this respect, the findings were similar to the earlier

ones reported by Lederberg (133a). However, in Stocker's material the evidence of motility within the subclones was best explained on the basis of the unilinear transmission of two characters: (*a*) the ability to produce motility-conferring particles, an ability presumably under the control of the motility gene being studied, and (*b*) the ability to travel in a liquid medium, an ability which depends on the presence of at least one hypothetical motility-conferring particle. The number of these particles produced in a cell containing the gene is calculated to be about 15, but their replication depends upon the continued presence of the gene. Particles distributed to a cell which does not receive the gene are eventually diluted out. The probable nature of the hypothetical particles will be discussed below.

Morse *et al.* (158, 159, 160) discovered an interesting type of transduction associated with the λ phage of *E. coli* K12. It differed from the system previously studied in *Salmonella* in that: (*a*) only one closely-linked group of bacterial markers, affecting galactose utilization (the Gal locus), was transduced by λ, and (*b*) λ phage transduces only when prepared by ultraviolet-induced lysis of λ-lysogenic strains. It had previously been shown by the Lederbergs (130) that λ prophage occupies a site on the bacterial chromosome adjacent to the Gal locus. A third difference resides in the fact that the transduced *E. coli* bacterium is often unstable, throwing off cells having the untransduced (Gal−) phenotype with a frequency of about $10^{-3}$ per bacterium per generation. Since the galactose-negative segregants are negative at the same chromosomal site as was the original untransduced strain, it is clear that the unstable transductants, or heterogenotes, had a fragment added rather than substituted in its genome. The Gal locus is complex, and several recombinable mutational sites have been recognized within it. By the study of recombination between markers present on the donor fragment and the host chromosome, it could be shown that the heterogenote will undergo one of the following processes: (*a*) The original heterogenotic condition may continue to be propagated; or (*b*) the heterogenote may undergo reduction to form haploid bacteria with markers of either the original recipient, the transduced fragment, or recombinants between the two; or (*c*) recombination may occur between host genome and homologous fragment with continuation of the heterogenotic state. The first of the three processes is the most likely event (occurring over 80 per cent of the time), the last occurring the least frequently.

An interesting finding (160) was that ultraviolet-induced lysates of heterogenotes contained phage particles nearly every one of which was capable of transducing the Gal locus (HFT phage), in contradistinction to the low frequency of transducing phages bearing a specific marker in ordinary transduction experiments. The question arises whether or not the genetic fragment bearing the Gal marker is the prophage itself. Weigle (221) provided evidence that the HFT phage is closely coupled with the Gal marker, by showing that, in ultraviolet-induced heterogenotes or in sensitive bacteria infected with HFT phage, multiplication of the Gal marker

occurs and in a manner similar to that of the phage itself. Campbell (*27*) has found that sensitive (nonlysogenic) recipients of *E. coli* K12, when infected at low multiplicity with HFT phage bearing the Gal+ marker, give rise most often to defectively lysogenic heterogenotes which lose immunity to superinfection with λ phage simultaneously with the loss of the Gal+ character. (The indications of defective lysogenesis were the inability to liberate phage upon induction and immunity to infection with λ phage.) Nonlysogenics stable (nonheterogenotic) for the Gal+ character also occur, but λ lysogenics are rare. At high multiplicity of infection of sensitive recipients with HFT phage, lysogenics are common and their Gal− derivatives are also lysogenic. High multiplicity of HFT phage is not the only way to obtain this latter effect for, if HFT phage are used at low multiplicity together with nontransducing phage (from ultraviolet-induced lysates of lysogenic Gal− strains) at high multiplicity, the same results are obtained. In the latter case, almost all transduced clones contain cells lysogenic for the *h* marker of the nontransducing phage. When a Gal− derivative appears, it is still lysogenic for the *h* marker. All of these findings can be explained, as Campbell points out, on the hypothesis that heterogenotes are the result of infection by defective phage, liberated by the ultraviolet-induced lysogenic *E. coli* K12, and that simultaneous infection with normal phage present in the same lysate increases the probability of establishing lysogeny (146, 150). Prophage carried by the heterogenote is not linked to the Gal marker in the "extra fragment"; rather, it is the defective prophage which carries this marker. This hypothesis is consistent with the results obtained by infecting lysogenic strains with HFT lysates. There is no effect of multiplicity, the efficiency of transduction being that obtained with sensitive recipients infected at low multiplicity. Presumably, the immunity conferred by the carried prophage prevents the nondefective phage from increasing the efficiency of transduction. Both the transduced clone and its Gal− derivatives are, as to be expected, lysogenic for the *h* marker of the original prophage. Substitution of the original prophage by phage carrying the *h* marker in the HFT lysate, and double lysogeny, occur but rarely.

### Lysogenic Conversion

The establishment of lysogeny in a sensitive bacterium is, in a real sense, a transfer of a hereditary determinant into the host genome. At least one significant heritable attribute is gained by the bacterium in this way: immunity to subsequent infection by the same or related phages. This property, however, is not easily dissociated from the prophage itself. The phenomenon of "zygotic induction" unequivocally demonstrates that the prophage is the sole determinant controlling lysogeny and that it is located at a specific site on the bacterial chromosome (115). If, during conjugation (to be discussed further), a portion of the bacterial chromsome containing the prophage passes from the lysogenic donor into a sensitive recipient, the prophage immediately enters the lytic cycle. Thus, lysis of the recipient can

be directly related to the length of the chromsomal segment it receives from the donor which, in turn, is measurable by the number of genetic markers introduced.

There are two properties of the prophage that would distinguish it, in any case, from a normal gene. First of all, it must be "loose" enough in its attachment to the chromosomal site to permit rapid transition when induced by ultraviolet light or other conditions (154), into a lytic cycle of growth. Second, it is known that different prophages occupy different loci, and that perhaps some prophages have variable, though preferred, attachment sites (17, 117). Fischer-Fantuzzi, Rita & Russi (64) found that two serologically related phages isolated from lysogenic *Shigella* strains occupy different sites on the chromosome of *E. coli* K12: one is closely linked as a prophage to λ and to Gal, while the other seems capable of attaching to different chromosomal sites. Jacob & Wollman (117) have made an extensive study of fourteen different prophages in *E. coli* K12, half of which are ultraviolet-inducible and half of which are not. The former group of seven can be located, by means of zygotic induction, recombination, and mechanical shearing techniques (to be described below), in a well-defined region of the chromosome, namely, between the Gal locus and the preferential rupture point (R) of the Hfr H strain. The other group of seven noninducible prophages are located beyond the rupture point (and are not transferred by conjugation into a sensitive recipient from a lysogenic Hfr H donor).

The foregoing discussion is hardly intended to be a comprehensive review of recent work on lysogeny [see (17)], but is intended to emphasize the highly interesting problem of the relation of prophage genetic material to bacterial genetic material. Earlier work had already suggested some interaction between prophage and bacterial genomes: (*a*) increased reactivation of ultraviolet-irradiated phage in irradiated bacteria, and the high frequency of mutants among the reactivated phages (220), and (*b*) nongenetic modification of the host range of phage as a consequence of growth in host cells of a particular strain or in a particular physiological condition (153).

The problem arises again in attempting to distinguish between transduced characters which are attributable to the prophage itself, transduced characters attributable to portions of the bacterial genome "linked" to the site of attachment of the prophage, and transduced characters which are attributable to portions of the bacterial genome fortuitously carried by phage. If, for example, the prophage of the classical *Salmonella* transduction experiments (235) has no fixed or preferred attachment site, but is capable of attaching at random, bacterial markers would be distributed in a random way among the phage particles released by lysing cells, as is observed. Perhaps it would be unwise to adopt a rigid attitude with respect to the taxonomy of phage-mediated genetic transfers; an underlying unity may exist for these processes.

An interesting case of phage-mediated transfer in which distinction

between bacterial property and prophage property becomes a semantic question is that of lysogenic conversion. Groman (86, 87) showed that the ability of *Corynebacterium diphtheriae* to produce toxins is strictly linked to the presence of β prophage. Acquisition or loss of this prophage resulted alternatively in gain or disappearance of the toxigenic character. Groman recently (88) has found that phage from certain naturally occurring nontoxigenic strains of *C. diphtheriae* can convert other nontoxigenic strains to toxigenicity. In four cases studied, the toxigenic strains thus produced were lysogenic for a phage indistinguishable from the previously described β-converting phage. The donor itself was never lysogenic and was not acted upon by the β-converting phage produced by the converted recipient. It is not yet clear whether recombination between related, nonconverting phages is the basis of the reconstitution of the β-converting phage in these strains. If so, it would be difficult to understand why the strains would be immune to β phage while receptive to a related phage capable of contributing markers to its reconstitution.

Sometimes a temperate phage will confer upon its lysogenized recipient bacterium the ability to produce an additional somatic antigen (217). Baron, Formal & Washington (7) have shown that a phage isolated from *Salmonella kentucky* will lysogenize certain other species of *Salmonella,* and in doing so bring about the production of a novel antigen. If the recipient cells are lysed rather than lysogenized, the lysates contain phage still capable of adding the particular antigen associated with its presence as a prophage. The frequency of cells with the new antigen among the survivors of exposure to this phage is considerably greater than the frequency of transduction ordinarily observed in *Salmonella.* All of these facts again indicate a marker closely associated with, or an integral part of, a specific prophage. Zinder (235a) studied another case of antigenic conversion brought about by lysogeny, that of the production of somatic antigen 01 in *Salmonella typhimurium* strain LT 2 by temperate phage P22. The presence or absence of antigen 01 in the bacterium was strictly linked to the presence or absence, respectively, of the prophage. Furthermore, no masked antigen 01 could be discovered in nonlysogenic cells, nor could any serological relationship be established between antigen 01 and any antigens in the phage. The new antigen could be detected within about 7 min. following infection, and appeared whether infection was caused by the temperate P22 or by any of its virulent mutants. Thus, conversion is not obligatorily associated with lysogenization. By means of crosses with a relative of P22 which does not cause production of antigen 01, it could be shown that the converting property resulted from a unit factor or gene mappable on the phage chromosome.

Finally, an old case deserves rementioning, that of the induction of thymine synthesis by T2 infection in the thymineless strain (T—) of *E. coli* 15 isolated by Barner & Cohen (4). It will be recalled that T— dies in the absence of thymine because of unbalanced growth, DNA synthesis stopping while RNA and protein syntheses continue. T2 phage can be adsorbed

slowly onto T− in minimal medium, and infection results in the normal production of phage DNA which contains the usual proportions of purine and pyrimidine bases, including thymine. How a phage particle can reactivate a genetically blocked synthetic process in the host is highly problematic, but it is worth considering that the phage genome possesses markers which, in cooperation with those of the bacterial genome, can regulate macromolecular synthesis. Perhaps even the DNA of virulent phage, immediately after infection, synapses with the bacterial chromosome, and switches metabolic processes along the pathway leading to phage multiplication and eventual lysis of the host.

## Conjugation

In recent years two important discoveries about the nature of recombination in *E. coli* K12 have revealed a fundamental similarity between this phenomenon and transduction and transformation. These discoveries concerned (*a*) the direction of transfer of genetic material, and (*b*) the quantity of genetic material transferred.

*Direction of transfer.*—By a variety of ways it has been shown that only one of the strains (F−) involved in conjugation receives genetic material (34, 98). The conjugants of the strain contributing the genetic material (F+ or Hfr) usually do not die in the process, but are fully viable after natural or experimental separation from the recipient bacteria (132, 136). Presumably, the continued viability of the F+ conjugant comes from the multinucleate condition of the bacteria. Nevertheless, in speaking of conjugation, it is justifiable to use the terminology already adopted for transduction and transformation, that of donor and recipient (or host) bacteria. F+ bacteria seem to differ from F— in certain surface properties, as reflected in electrophoretic mobility (155) and antigenic properties (140). Anderson *et al.* (2) have obtained with the electron microscope some striking photographs of conjugating pairs. These pictures reveal a small amount of the wall of each of the conjugants forming a continuous bridge between them. Perhaps because of the difference in their surface properties, F− is more motile than F+ (200), so that in the course of selecting for high motility in F+ strains, the selected clones are almost always F−.

The donor characteristic of F+ is generally transferred to the F− exconjugant, regardless of whatever other markers are incorporated into the F− genome (98). The electron microscope studies of Anderson *et al.* indicate that, under conditions where F− cells are in excess, the efficiency of conjugation of an F+ bacterium is as high (*ca.* 100 per cent) as that of an Hfr bacterium in an Hfr × F− cross, which yields a high frequency of recombinants ($10^4$ times greater than that of F+ × F−). Wollman, Jacob & Hayes (227a) suggest that the F+ characteristic does not result from a determinant essentially different from any of the others transferred (for example, a cytoplasmic virus as opposed to a chromosomal gene), but arises from a marker having a high probability of transfer through the conjugation bridge. According to their view of conjugation (see below), this

would be a marker very close to the initial portion of the F+ chromosome which passes in an oriented way into the F− cell. The only difference between an Hfr and an F+ strain would be that the length of the chromosomal segment capable of being transferred by the latter is considerably smaller and contains, so far as one knows, only the determinants for the F+ and colicinogenic properties.

There appear to be several different F+ agents (138). Furness & Rowley (74) have found that a number of wild-type *E. coli* strains are able to convert *E. coli* K12 F− into F+ without at the same time contributing any of the other markers studied. Yet, all do not convert the same K12 F− mutants, and their relative efficiency in transferring the F+ factor varies among them. Bernstein (16) obtained similar results in a study of two different F+ agents, the one originally found in the K12 strain and another found in the WG3 strain of *E. coli.*

Hfr mutants, which may be defined as donor strains which contribute with high efficiency markers other than the F marker, have been obtained in the past as the result of spontaneous mutation in an F+ culture [Hfr H, (98)] or of treatment of the F+ culture with nitrogen mustard [Hfr C, (31).] An Hfr strain is characterized by a high frequency of transfer of a specific length of the bacterial chromosome, markers not on this segment being transferred with the low frequency characteristic of an F+ strain. Jacob & Wollman (116) have proposed a hypothesis which states that all transfers of genetic material during conjugation are made by Hfr cells, and that the low frequency of recombinants obtained with F+ cultures result from the content of spontaneous Hfr mutants rather than to a low probability of transfer characteristic of each F+ cell. A statistical fluctuation test did indeed show that the number of recombinants varied in a random way when the F− bacteria were exposed to different samples of the same F+ culture, but the variance was considerably greater than the mean when samples from different F+ cultures were used. The variance in the latter case was what would be expected if F+ cells undergo rare spontaneous mutations to Hfr. By indirect selection (replica plating) Hfr mutants were eventually isolated from F+ cultures. In comparing a number of independently-isolated Hfr mutants, each selected for high frequency of transfer of some particular marker, Jacob & Wollman found that they had selected for different Hfr mutants which could be distinguished with regard to the groups of markers they transferred with high frequency.

Furness & Rowley (74) and Bernstein (16) also observed that F+ agents differed, not only in respect to the efficiency of F+ transfer, but also in respect to efficiency of transfer of the unselected markers in a cross. These results may be explained, by the Wollman-Jacob hypothesis, as arising from different kinds of Hfr mutants present in different cultures and different strains of *E. coli.* Bernstein (16) also reports that the donor-recipient relationship does not seem to occur in crosses with the F+ agent from the WG3 strain of *E. coli.*

One peculiarity common to all (six) of the Hfr strains studied by Jacob & Wollman (118) is the inability to transfer either the Hfr or the F+ characters. It is not clear why the F+ → Hfr mutation should always result, in the absence of the F+ marker, within the group of markers capable of being transferred with high frequency. Presumably the original F+ strain is, in actuality, an Hfr strain capable of transferring only F+ (and the determinant for colicinogenesis) with high frequency. Richter (182) has obtained, by ultraviolet irradiation of an F+ culture, a new Hfr strain ($Hfr_3$) which apparently can transfer the F+ determinant with high frequency. This $Hfr_3$ marker was found to be linked to a locus concerned with maltose-utilization (Mal) and, in this respect, was different from the Hfr markers in the Hfr C and Hfr H strains. The F+ determinant seems, however, to move in its position on the chromosome from a site close to $Hfr_3$ to another (or other) site(s) not linked to $Hfr_3$. This was indicated by the fact that although the F+ bacteria obtained in the $Hfr_3$ × F− cross (10 to 20 per cent) were no longer $Hfr_3^+$, a high proportion of the $Hfr_3^+$ segregants (0.01 per cent) were also F+. Furthermore, the $Hfr_3$ strain oscillated between a highly (F+) infective and a noninfective state. Possibly the Hfr C, Hfr H, and Hfr mutants obtained from F+ by indirect selection are different in that the F+ agent is stably fixed to a noninfecting Hfr locus in these strains (33).

That the F+ agent has properties not shared by other chromosomal markers, is evidenced not only by Richter's findings of the instability of F+ with regard to its chromosomal site in $Hfr_3$, but also by the observations of Hirota (100) that F+ cultures can be converted into F− by treatment with cobalt or nickel. No other characters of the F+ bacterium appear to be altered by this treatment, the frequency of the converted F− cells being about $10^5$ greater than that of other mutants. The conversion occurs in the absence of cell multiplication, and both F+ and F− strains are equally sensitive to cobalt killing, so that selection of F− mutants cannot be the mechanism of the change. Furness & Rowley (74) have also been able to separate colicinogenicity and the F+ character by means of cobalt treatment.

*Quantity of genetic material transferred.*—Two explanations have been offered for the fact that only certain markers are transferred with high frequency by a particular F+ or Hfr strain. According to Lederberg (132), an entire chromosomal complement of markers is transferred in such a cross, but a certain segment of the F+ chromosome is eliminated by the zygote following synapsis of host and donor chromosomes. This view is based upon results obtained with diploid strains occasionally produced after an F+ × F− cross (164). These strains are hemizygous for a segment of the chromosome bearing two loci, Mal and S. Eighty per cent of the diploid progeny were found to contain the alleles of the F− parent on this segment, while 20 per cent contained the alleles of the F+ parent. On the assumption of defective synapsis and postzygotic segmental elimination, Cavalli-Sforza & Jinks (33) were also able to map, on a single chromo-

some, all known *E. coli* markers (those transferred at high and at low frequency in an Hfr × F− cross). The second explanation comes from Wollman, Jacob & Hayes (227a), who state that only part of a donor chromosome is ever transferred. Elimination of part of the chromosome, therefore, is prezygotic in their view, and the zygote is incomplete (a merozygote). The amount of genetic material transferred depends upon the particular Hfr mutant involved in the cross.

A considerable weight of evidence favors the latter explanation as can be seen from the following points. First, if, at various times after effective contact is made between Hfr and F− conjugants, the culture is shaken, or streptomycin or phage is added to which the Hfr parent alone is sensitive, one finds that the number of Hfr markers which can eventually recombine increases with increasing time between the contact of conjugants and treatment (227a). Furthermore, there is an order of transfer as evidenced by the fact that certain markers are transferred early and others progressively later, until, at a certain time, all markers capable of recombining at high frequency are transferred. The sequence of markers in the transferred segment corresponds to the linkage map which is constructed from the recombination data in a control Hfr × F− cross. It seems unlikely that some postzygotic elimination process is affected in the same way by these different kinds of treatment. Rather, it seems more likely that the penetration of the donor chromosome into the F− recipient is interrupted in each of these treatments by removing (or destroying) the Hfr parent. Secondly, if the Hfr parent is lysogenic for λ and the F− parent is sensitive, a certain proportion of the F− cells are induced to lyse (zygotic induction) as a result of penetration by that portion of the Hfr chromosome bearing the λ prophage (115). In such a cross the frequency of recombinants containing a particular Hfr marker depends on the linkage distance between that marker and the site of attachment of λ prophage: the greater the distance the more frequent are the recombinants containing that marker (227). These facts are readily explained on the basis that λ prophage immediately enters the lytic cycle upon penetrating a sensitive F− host, that most of the markers precede it during the oriented penetration of the Hfr chromosome, that spontaneous breaks occur during penetration, and that the probability of penetration of a marker is a function of the distance between that marker and the point of the chromosome which initiates penetration. From the frequency of zygotic induction per Hfr bacterium involved in the cross (> 50 per cent) and from the reduction of the frequency of recombinants below that observed in a cross where zygotic induction does not occur (for example, where both Hfr and F− are λ-lysogenic), it can be calculated that nearly 100 per cent of the Hfr cells participate in conjugation (where F− is in excess). The third line of evidence follows: Hfr bacteria may be labeled with $P^{32}$ and stored in the cold for various lengths of time to allow for the decay of different amounts of $P^{32}$ atoms. If Hfr bacteria, after these various times of storage, are allowed to conjugate with unlabeled F− in a

nonradioactive medium, it is found that the ability of these Hfr to form recombinants of any type falls off exponentially as a function of $P^{32}$ decay. The rate of loss of a marker depends on its position on the Hfr chromosome. The farther it is from the presumed initial point of penetration, the greater its rate of decay (73, 118). When the slopes of the decay curves for the various markers are compared, they are found to have the same values relative to each other as those of the relative times of penetration determined in the previous experiments. It appears that $P^{32}$ decay, occurring at random and with equal effect all along the Hfr chromosome, can prevent transfer of genetic material, probably by breaking the DNA chain, and the probability of prevention is greater the longer the segment to be transferred.

The $P^{32}$ experiments made by Fuerst *et al.* suggest strongly that DNA is transferred during conjugation, just as it is inferred that DNA is transferred in transduction (75). If radioactive Hfr bacteria are mated with unlabeled F— in a nonradioactive medium and then stored in the cold for various lengths of time, it is found that the segment contributed by the Hfr parent remains sensitive to $P^{32}$ decay even after transfer (73, 118). However, the period of sensitivity lasts only 2 hr., indicating that by that time the genetic information brought in by the transferred segment of the Hfr chromosome has been turned over to some nonradioactive material. The main effect of $P^{32}$ decay in the transferred segment is to decrease the probability of multiple markers being incorporated in the postzygotic period. The rate of loss of a given marker as a function of $P^{32}$ decay appears to be the same as for any other marker, but the rate of loss of multiple recombinants is much greater. The same effect was observed with the ultraviolet-irradiation of the Hfr bacteria prior to mating. Thus, both ultraviolet and $P^{32}$ decay appear to interfere with genetic incorporation of the transferred Hfr segment.

In comparing the linkage relationships of the markers introduced by the different Hfr mutants, which they isolated, Jacob & Wollman (118) noted that while the extent of the segment transferred at high frequency varied from one Hfr strain to another, the distances between the markers on the segment remained the same. However, the polarity of penetration varied between Hfr strains, so that with one strain the marker for resistance to phage T6 may enter early and with another strain it may enter late. The only logical way of mapping the K12 markers was on a circular chromosome, since it was not possible to determine the ends of the chromosome. These results account for the findings of Skaar & Garen (199) who, using the technique of interrupting transfer by destroying the Hfr parent with virulent phage at various times after mating, observed an order of genes on the chromosome of the Hfr C strain similar to that for the Hfr H strain, but with a reversed polarity of penetration. Belser & Bunting (12) reported a process of genetic transfer in *Serratia marcescens* resembling that of conjugation in *E. coli* K 12. Cell-to-cell contact appeared to be essential for transfer, and a donor-recipient relationship was also observed. Incomplete transfer

also appeared to be the rule, since linked transfers were exceptional. In view of recent findings, we might explain conjugation in *Serratia* as the result of extreme heterogeneity in Hfr mutants within the F+ population.

Fisher has shown that zygote formation in *E. coli* K12 depends upon a supply of free energy made available by oxidation via the Krebs cycle (65, 66). The addition of glucose and a Krebs cycle intermediate (aspartate or succinate, etc.) considerably increased the number of zygotes formed. The energy was required uniquely by the Hfr parent, and only during the period of transfer. Inhibitors of the Krebs cycle, but not specific inhibitors of DNA, RNA, or protein synthesis, reversed the effect of the added glucose and the Krebs cycle intermediate. Hayes (99) showed further that the rate of zygote formation depends upon the temperature. In part, the temperature affects the frequency of collision between mating cells, but in part it regulates the speed of chromosome transfer, presumably by affecting the rate of energy release through aerobic oxidation. Reduction in the temperature from 37° to 32°C. doubles the time required for any Hfr marker to enter the F− cell. This finding suggests that the penetration of the Hfr chromosome occurs at a continuous rate. Changing the pH of the medium or the density of the conjugating cells did not alter the times of penetration of the various Hfr markers, although they did affect the rate of effective contacts between the mating bacteria. Hayes also studied the rate of appearance of recombinants possessing the marker for azide resistance or the marker for resistance to phage T1, and found that azide resistance is dominant to sensitivity inasmuch as expression is complete before the transferred marker is segregated into a haploid clone. Resistance to phage T1, on the other hand, does not begin to be expressed until segregation has occurred and requires four further generations of growth for completion.

Lederberg (136) and Anderson & Mazé (1) have begun experiments in which the pedigrees from single Hfr × F− zygotes were studied. Mating pairs were isolated with a micromanipulator, and the exconjugants, which usually separated spontaneously after an hour or two, were allowed to divide. The cells produced after a few fissions were then separated and allowed to produce clones which were tested for phenotype by plating on appropriate media. The results of these workers were essentially quite similar. Both noted that recombinants were found only among the progeny of the F− exconjugant (the zygote), which would be expected from unidirectional transfer of genetic material. Anderson & Mazé observed irregular division of the zygote, and decreased viability and altered morphology fairly common among its descendants. Both workers noted a prolonged delay in the segregation of an Hfr marker into pure clones; the delay was always longer ($> 5$ generations) than that expected from the separation of the four "nuclei" present in the zygote (2 generations). Lederberg noted that in none of the subclones (produced by daughters following the first to sixth fission) was there any evidence of a complete Hfr genome. As he pointed out, this could come from either partial transfer, as hypothesized by Jacob

and Wollman, or from elimination of a segment of the Hfr chromosome before the first division following transfer. It is interesting to find that conjugation, transduction, and transformation share a substantial delay in segregation of the transferred marker.

Kirchner & Eisenstark (125) have made the interesting observation that removal of divalent cations from the medium increases the frequency of recombination in *E. coli* K12 and of transduction in *Salmonella typhimurium*. The effect occurred only when recipient cells were starved of divalent cations, never when the donor cells were so treated. It appears as though transfer was not affected but that incorporation was, possibly through breakage of the transferred chromosomal segments. Future work should be able to test this notion.

Ogg & Zelle (169) isolated a camphor-induced mutant from a purine-requiring strain of *E. coli* B/r. The mutant showed increased cell size, increased radiation resistance, and increased DNA and RNA content per cell. It was converted to F+, and the F+ and original F− strains were used in reciprocal crosses with suitable markers (234). The increased cell size and radiation resistance segregated as a unit in these crosses, and the segregation was influenced by F polarity as does that of unselected markers in general. It could not be determined whether the mutant characteristics resulted from a polyploid condition or from a single gene present on the transferred segment of donor chromosome.

Luria & Burrous (152) have described the results of their investigations on conjugation between strains of *Shigella* and *E. coli*. In view of their relevance to considerations of bacterial evolution, they will be discussed under section IV below.

### Heterokaryosis

The process of hyphal anastomosis resulting in heterokaryosis is common among molds. Sermonti & Spada-Sermonti (196) described such a process occurring also among the streptomycetes. By the use of several markers, recombination was reported to have taken place. Bradley (20) has recently reported that heterokaryosis may be carried through the spores of *Streptomyces coelicolor*. Prototrophic heterokaryosis produced from two mutant mycelia produce spores which dissociate into the original parental types. Possibly because of the close linkage of the markers used in his studies, Bradley was unable to find any evidence of stable prototrophs resulting from recombination. He did find some spores which showed a parental mutant phenotype at first, but which reverted to prototrophy. These spores were multinucleate, and their phenotype could well depend on the proportion of nuclei of different types contained within them.

Braendle & Szybalski (21) have studied heterokaryosis in seven different species of *Streptomyces*. They found that, although heterokaryosis occurred between mutants of the same species, it did not occur between mutants of different species. In two species, *S. coelicolor* and *S. fradiae*, fusion of unlike nuclei (synkaryosis) was observed to take place in the heterokaryon

followed by genetic recombination which was detected by the production of conidia of the recombinant types. These authors found that the frequency of the recombinant conidia varied considerably, depending upon the markers employed, possibly because of the operation of nuclear selection within the heterokaryon between the time of synkaryosis and the scoring of recombinant colonies.

### Infection of Protoplasts

Protoplasts are viable forms of bacteria, although incapable of multiplication, in which cell wall synthesis appears to be blocked (135, 176). They usually assume swollen, spherical shapes, and are highly sensitive to osmotic changes although fairly stable in hypertonic sucrose media containing $Mg^{++}$. Formation of protoplasts may be induced by lysozyme (236), by penicillin (133), or by mass adsorption of inactivated phage (29). If infected by phage prior to protoplast induction, the biosyntheses normally involved in phage growth will continue (236). Enzyme synthesis can also be induced in protoplasts (204) so that, clearly, they are capable of important macromolecular synthesis. Spizizen (205) observed that disrupted T2 virus preparations possessing no measurable infectivity nevertheless caused *E. coli* protoplasts to produce infectious T2 virus. The ability of protoplasts to produce phage did not depend on whether the normal strain was sensitive or resistant to the intact virus. Spizizen believed that removal of protein from the disrupted virus preparation resulted in loss of phage productivity in the treated protoplasts. Fraser *et al.* (71) found that DNA preparations obtained from phage T2 treated with 8 *M* urea were capable of infecting *E. coli* B or B/2 (resistant to phage T2) and causing the production of T2 phage. They found, however, that contrary to Spizizen's findings heat and versene treatment had little effect on the activity of the DNA preparations although DNase inactivated it rapidly. Fraser *et al.* point out that their methods of preparing DNA (by urea treatment rather than by osmotic shock) are different, as is their method of preparing protoplasts. "Intact" cells seem to be very slightly infectable by DNA. This was true of the phage-sensitive (B) and phage-resistant (B/2) strains, which suggests that this slight infectability of "whole cells" is due to "spontaneous" protoplasts present in the cultures. Interestingly enough, in view of the known importance of serum albumin in promoting adsorption of DNA in transformation (see above), it was found that beef serum albumin stabilizes protoplasts.

These findings, albeit preliminary, on the infection of protoplasts by viral DNA raise enormous possibilities for future research. Perhaps the protein coat of the bacteriophage is essential primarily for adsorption onto the bacterial wall and for converting a localized area of the bacterial surface into that of a "protoplast." If viral DNA can infect protoplasts, why not bacterial DNA? Chargaff, Schulman & Shapiro (35) have offered some evidence that protoplasts of *E. coli* strain W can be transformed by DNA. Impure DNA preparations were used to avoid major degradation of the DNA. A lysineless strain (lys−) was treated with the DNA extracted from

the wild-type (lys+) strain, and in several experiments lys+ were recovered at a higher frequency than in the absence of DNA or when whole cells were tested in the presence or absence of DNA. Unfortunately, several controls were not performed, such as those concerning the sensitivity of the transforming preparation to DNase, as well as the transforming specificity of the preparation. The significance of transforming protoplasts with DNA is so exciting that further work in this field is surely indicated.

## FUNCTION OF GENETIC MATERIAL

### Position and Function

The genetic material of the chromosome is divisible into units separable by recombination. In the division of the chromosome, loci are recognized within which mutations result in very similar phenotypic changes as, for example, losses in the ability to carry out the same biosynthetic reaction. The question arises whether these mutational sites within loci are distinguishable in any functional way, other than separability in recombination. For the study of this question, genetic studies of bacteriophage (14) and of bacteria have been very helpful.

By transduction techniques, Demerec and his colleagues (46, 47) have been able to establish that about 400 auxotrophic strains of *Salmonella* could be attributed to mutations at 42 different loci. A number of loci (ranging from 2 to 5) often affected the synthesis of the same growth factor. In general, mutations at these different loci resulted in blocks at different steps in the biosynthesis of the metabolite in question. In a cross (infection of a recipient strain by phage liberated from a donor strain of different genotype) in which the loci involved were different, the expected prototrophs appeared. Prototrophs also appeared, however, in crosses between donor and recipient bacteria sharing the same biochemical block. These were attributed to recombination between nonidentical mutational sites within the same chromosomal region or locus. The frequency of recombination varied with the particular alleles or markers involved in the cross, and these markers could be ordered in a linear array because the recombination frequencies between different pairs were additive. In 36 of the different loci identified, two or more mutants were available for study. In each of these cases, either all or most of the mutants were found to be attributable to nonidentical mutated sites within the same locus (nonidentical alleles in Demerec's terminology, or pseudoalleles).

There is some evidence for differentiation in the biochemical functioning of nonidentical alleles (94, 229), for sometimes they can be distinguished by various phenotypic effects; for example, in respect to modifiability of the growth factor requirement under certain environmental conditions. Moreover, nonidentical alleles having similar properties tend to be grouped together within the locus.

A few cases of independently obtained mutations at the same locus appeared to involve the same chromosomal site (identical alleles in Demerec's

terminology), in that extensive tranduction tests (in which recombinants occurring with a frequency of about $10^{-8}$ could be detected), failed to produce prototrophic recombinations between them. Hartman (94) has evidence for three pairs of such apparently identical alleles involved in the same step in the biosynthesis of histidine. Yet, only one pair was phenotypically nondifferentiable. The other two pairs were differentiable either in rates of spontaneous mutation, of ultraviolet-induced reversions, or of recombination with other markers in the same locus. The question arises whether these sites, capable of different states, are the ultimate discrete units of organization of genetic material. Yura (229) also has evidence for phenotypically differentiable, identical alleles in a locus concerned with adenine synthesis. Starlinger & Kaudewitz (206) have found a suppressor mutation at another locus which permits one of the histidine-requiring mutants to form colonies, albeit smaller than those of wild-type, on minimal medium. It appears that this suppressor acts specifically on one of twenty-six different histidineless mutants. Moreover, in its reaction to the suppressor gene, this mutant can be distinguished from five other nonidentical alleles at the same locus. This observation is regarded as evidence for the view that the sites separable by recombination are, indeed, sites of specific genetic function.

A class of mutations was discovered [see (46)] at several different loci that possessed the following characteristics: (*a*) failure to produce prototrophic recombinants with a number of closely linked markers; (*b*) absence of spontaneous reversion to prototrophy; (*c*) rarity of this class relative to the frequency of the class of recombinable intralocus mutations; (*d*) low frequency of crossing-over with those adjacent markers with which a mutation of this type can recombine. This class of mutation is readily explained as a deletion of a well-defined region of the chromosome, usually restricted to a segment within a locus. In one case, however, studied by Clowes (36), such a multiple-site mutant, as it has been termed, appears to extend over most of one locus and part of another. The biochemical block of this mutant involves that of both loci (the ability to convert sulfate to sulfite, and the ability to convert sulfite to thiosulfate, in the synthesis of cysteine). Thus, in this case, there is a good correlation between the genetic evidence for the extent of the damaged (deleted?) region and the biochemical evidence on the extent of the biosynthetic block sustained by the mutant. Skaar (198), on the other hand, has found some tryptophan-requiring mutants which lack two enzymatic specificities and are revertible and, hence, seem to be the result of "point" mutations rather than to deletions. Further evidence to be discussed below indicates that the loss of two enzymatic specificites as a result of a single gene mutation may not be uncommon. Thus, there is no way at present for discriminating between a deletion extending over a chromosome segment having a number of functions and a "point" mutation resulting in a multiple deficiency.

Ozeki (170) observed that abortive transductions did not occur between

nonidentical alleles at the locus he studied. Skaar (198) has utilized this observation for making cistron assignments to different mutations at a locus affecting tryptophan synthesis in *E. coli* B. Members of the same cistron do not give a wild-type phenotype when in the *trans* position (one marker present on the host genome, the other marker present on the abortively transduced fragment). Morse, Lederberg & Lederberg (160) have also applied the *cis-trans* test to the Gal markers transduced by λ phage in *E. coli* K12. Some allelic combinations gave a galactose-positive (wild) phenotype in the *cis* or *trans* position, whereas others gave such a phenotype only in the *cis* position. When the *cis-trans* test can be supplemented with recombination data giving the precise order of the markers in question, it is hoped that a model relating the position of mutational sites to their functional organization can be constructed for bacteria as Benzer (14) has done for bacteriophage.

One of the surprising findings of the work of Demerec and his colleagues is the nonrandom arrangement on the bacterial chromosome of loci affecting related reactions in a biosynthetic pathway. In at least two cases (48, 94), one involving four tryptophan loci, the other involving four histidine loci, the loci are arranged in the same order as the steps they govern in the chain of biochemical reactions. Skaar (198) has confirmed this finding with a number of tryptophan loci in *E. coli* B. It is hardly likely that such an arrangement of loci would be maintained if it had no selective advantage. Perhaps, as Demerec has pointed out (47), such nonrandom distribution of loci controlling related biochemical reactions is of especial importance only in bacterial metabolism, for it is not observed in the case of *Neurospora* or other higher organisms.

## Gene Control of Protein Specificity

Assuming that DNA in some way imparts specificity to proteins at the time of their formation, an important problem is that of coding. How can the sequence of nucleotides, of which there are generally four species in a DNA chain, determine the sequence of amino acids of which there are a great many species, in a protein chain? Recently, Brenner (22) and Crick, Griffith & Orgel (43) have considered this problem. The former has ruled out the possibility of certain models (overlapping triplet codes), whereas the latter workers find that another type of model (nonoverlapping triplet codes, where each triplet specifies an amino acid), predicts an upper limit of 20 for the number of amino acid species the code would be able to handle.

The route by which the information inherent in a given DNA structure is eventually passed to a protein-synthesizing system may very well be indirect and even complex, and there are several lines of evidence to indicate that RNA serves as an intermediary storage of this information. Since discussion of this evidence would take us beyond the bounds of this review, the reader is directed to recent reviews by Spiegelman (203, 204) and articles by Vogel (218, 219). Relevant also are recent studies by Bećarević

(9, 10, 11) on the relation of ultraviolet inhibition of RNA synthesis to ultraviolet inhibition of induced catalase synthesis in yeast. Although RNA synthesis appears to occur independently of the presence of a nucleus in the large unicellular alga, *Acetabularia* (208), there is supporting evidence for the view that this is not typical in microorganisms. Prescott (175) has shown that enucleated halves of amebae can take up $C^{14}$-uracil, but cannot incorporate it into RNA, as nucleated halves can.

The pedigree evidence obtained by Stocker (209) in abortively transduced clones, and by Quadling & Stocker (178) in "phenotypic excursions" within nonmotile clones of *Salmonella,* has indicated two distinct systems at work in the synthesis of flagellar proteins. The first of these is the gene-controlled synthesis of motility-conferring particles, and the second is the synthesis of flagella by these particles. The lack of identity between the motility-conferring particle and the flagellum itself was suggested by the calculation that each bacterium containing an abortively transduced gene is capable of producing 15 motility-conferring particles. This number is larger than the average number of flagella per bacterium in a motile transductant. It is possible, however, that the hypothetical motility-conferring particle is the basal granule that secretes the flagellum and that there is a delay between the formation of a granule and the production of a visible flagellum. The synthesis of new flagellar proteins appears to be retarded by growth at elevated temperatures (179). At 44°C. new flagella formation is stopped, and the already formed flagella are shared about equally between the daughter cells produced by fission, until progeny are produced with either one flagellum or none.

However indirect the heterocatalytic function of a gene may be, the evidence is nevertheless increasing that the result of gene mutation, at the molecular level, is an altered specificity of a protein. Fincham & Boylen (62) have studied four mutant strains of the mold *Neurospora crassa,* which had been previously shown by Newmeyer (165) to result from very closely linked, but separable mutations. These strains are all characterized by the inability to synthesize arginine and to respond to citrulline, and by their accumulation of argininosuccinic acid in the medium. Although an argininosuccinase capable of catalyzing the splitting of argininosuccinic acid to arginine and fumaric acid could be demonstrated in purified cell-free extracts of the wild-type mycelium, no enzyme activity could be detected in either crude or purified preparations from the mutants. The sensitivity of the test for enzyme was good enough to detect 1 to 2 per cent activity in crude preparations and 0.2 per cent in purified preparations. Argininosuccinase activity of wild-type preparations was not inhibited by additions of mutant extracts, nor by simultaneous extraction of mixed wild-type and mutant mycelia.

Fincham & Pateman (63, 172) have also studied a complex locus concerned with the production of glutamic acid dehydrogenase in *Neurospora.* Several recombinable mutations at this locus result in loss of ability to pro-

duce detectable enzyme and consequent inability to grow on minimal medium. Production of wild-type enzyme is restored by recombination of different mutations, although the rate of recombination is very low (0.001 to 0.003 per cent). This appears to be a case of distinct mutations at different sites of a complex locus, each resulting in the altered production of the same specific enzyme. The interesting feature is that heterokaryons in which different mutations are borne in separate nuclei can produce from 15 to 50 per cent of wild-type enzyme activity. It appears as though the complementary action of different sites within the locus can extend not only between chromosomes, but also between nuclei. One mutation in this locus was obtained by ultraviolet. The strain bearing this mutation differed from the other mutants of this locus in that it grew almost as well as wild-type on minimal medium at 25°C., but required an exogenous supply of amino nitrogen at 20°C. or lower. The enzyme extracted from this mutant strain differed from that of wild-type in that it had little activity at 20°C., but could be activated by mild heat treatment or incubation with substrate. The heat activation of the enzyme was reversible. The extract did not appear to contain a heat-labile inhibitor of the enzyme inasmuch as addition of the mutant extract to a wild-type preparation did not result in inhibition of the latter. This mutation showed complementary action with one other mutation at this locus, but not with a second.

Giles, Partridge & Nelson (77) observed similar results with several mutations of independent origin at a *Neurospora* locus concerned with the synthesis of adenylosuccinase. Wild-type recombinants can be obtained at a low frequency in crosses involving different mutations at this locus. One of the mutants is temperature-sensitive and produces a thermolabile adenylosuccinase (at least in crude extracts). By means of the back-mutation test described above, it was shown that certain of the mutations at this locus are capable of reverse mutation to wild-like phenotypes. The reverted strains produce adenylosuccinase, but at levels or with stabilities less than those of wild-type. These workers also observed complementary action of certain pairs of mutations contained in separate nuclei within a bikaryon. Adenylosuccinase activity was found in extracts from these bikaryons. They have suggested the hypothesis that, in the case of complementation, two nonoverlapping damaged sites at this locus produce complementing templates that may interact in the cytoplasm; in the case of noncomplementation, the damaged sites of the locus overlap and their templates cannot interact successfully in the cytoplasm to produce enzyme.

Horowitz & Fling (102) have studied heterokaryons between two strains bearing different mutations at a locus concerned with tyrosinase production. One mutation, $T^s$, causes production of a thermostable enzyme; the other, $T^L$, results in production of a thermolabile tyrosinase. Extracts from the heterokaryons contained both forms of the enzyme, thus indicating that, in this case, the two damaged sites act independently of each other. It was also shown, by hyphal tip transfer and reconstitution of homokaryotic lines

from the original heterokaryon, that there was no cytoplasmic transmission of the ability to produce tyrosinase. Of six homokaryotic derivatives, three were shown to have all the characteristics of a $T^S$ mutant, while the other three were like $T^L$.

There is now good evidence to prove that some altered enzymes produced in mutant strains are inhibited by normal constituents of protoplasm. Suskind & Kurek (211) have studied the $td_{24}$ mutant of *Neurospora crassa*, which makes a temperature-sensitive tryptophan synthetase. Although no enzyme activity is found in crude extracts, it appears at about 30 per cent of the level found in wild-type extracts when purified with ammonium sulfate, protamine sulfate, or alumina gel. The activity disappears again, and in a stoichiometric fashion, if crude extract is added to the purified preparation. The inhibitor is found in wild-type extracts and in extracts of several mutants of the td locus, but only the enzyme produced by strain $td_{24}$ has a high susceptibility to it. The properties of the inhibitor suggest that it may be a metal.

Some biochemical work has been done on the complementary action between pigment-mutants of *Serratia marcescens*. When two of these abnormally pigmented strains are grown together, one is able to develop the red pigmentation typical of wild-type. Green & Williams (83) have found that the pigment produced syntrophically is exactly like the wild-type prodigiosin. Santer & Vogel (189) have confirmed this finding and have shown, by radioactivity experiments, that the "feeder" mutant accumulates a pyrrole-containing substance which is incorporated into the prodigiosin synthesized by the other mutant. Lewis (149) has described several new white variants which can be differentiated with respect to their ability to make prodigiosin from diffusible precursors elaborated by the "feeder" strain.

Some new work has been done on genetic control of adaptive enzyme synthesis. Lester & Bonner (145) have shown that the ability to utilize raffinose by *E. coli* is under the control of at least two distinct gene loci, one controlling α-galactosidase formation, which can be induced by melibiose, and the other controlling the constitutive synthesis of β-galactosidase. Rickenberg (183) has made protoplasts of a strain of *E. coli* K12 that cannot make β-galactosidase but which can make a permease that accumulates the galactoside inducer of this enzyme within the cell. The protoplasts had the same permease activity as intact cells, indicating that the cell wall is not the site of this enzyme. Englesberg (55) has recently described a mutation in *Salmonella typhimurium* LT2 that results in loss of ability to utilize glucose. The utilization of citrate by a strain containing this mutation is inhibited by glucose. Another mutation can occur which will relieve the specific inhibition by glucose. Acquisition of this latter mutation by the wild-type (glucose and citrate-utilizing) strain results in the disappearance of diauxic growth in media containing both glucose and citrate.

Suskind and Yanofsky are studying the relation of enzymes, the syntheses of which can be altered by gene mutation to other proteins produced

by the cell. A mutant of *Neurospora,* $td_2$, lacking tryptophan synthetase, which catalyzes the conversion of indole to tryptophan, nevertheless produces a protein (CRM) that is immunologically indistinguishable from the enzyme. Suskind (210) has shown that antibodies produced against CRM are as effective against tryptophan synthetase as antibodies produced by injection of purified enzyme. The enzyme and CRM can be distinguished, however, by their different sensitivities to dialysis; dialysis treatment not only inactivates the enzyme irreversibly but also affects its combination with antibodies. Dialysis treatment does not so affect CRM. Wild-type extracts appear to contain both tryptophan synthetase and CRM. Lerner & Yanofsky (144) have also detected a cross-reacting protein in mutants of *E. coli* which lack tryptophan synthetase. However, it appears to be absent in mutants blocked at other steps in tryptophan synthesis (anthranilic acid →indole glycerol phosphate →indole). This finding suggests that CRM, at least in *E. coli,* may be an enzyme involved in some terminal step in indole synthesis (perhaps between indole glycerol phosphate and indole).

Hotchkiss & Evans (111) have indicated a very fruitful approach to the correspondence between changes in the fine structure of DNA and changes in the fine structure of a specific enzyme protein. Three linked markers affect resistance to sulfa drugs in *Pneumococcus.* Each marker alone and each of the seven possible combinations produces a characteristic level of resistance. All three markers appear to affect the synthesis of a single enzyme involved in the utilization of *para*-aminobenzoic acid in folic acid synthesis. The activity of this enzyme can be followed by measuring synthesis of folic acid. The enzyme system is sensitive to sulfanilamide, and its degree of sensitivity is directly correlated with the degree of sulfanilamide sensitivity of the strain (which, in turn, is determined by the combination of the three DNA markers it possesses). The enzyme systems from strains containing different combinations of the genetic determinants for sulfanilamide resistance also show differences in their affinities with analogues of sulfanilamide (sulfaguanidine, *p*-nitrobenzoate and *p*-aminosalicylate). This work suggests that regions closely linked on the DNA molecule may determine various aspects of the specificity of a given enzyme protein or of a related group of proteins.

There is some evidence to indicate that a single mutational event may affect the production of two different enzymes. Englesberg (53, 54) has found that when wild-type *Pasteurella pestis* (R−) mutates as a result of a single event to the raffinose-utilizing (R+) condition, it acquires the ability to synthesize both rhamnose isomerase (rhamnose → rhamnulose) and rhamnulokinase (phosphorylation of rhamnulose). Neither enzyme is found in wild-type (R−) grown in the presence or absence of either rhamnose or rhamnulose, nor has any soluble inhibitor been found in either wild-type or unadapted R+ extracts which affects rhamnose isomerase or rhamnulose kinase. An alternative explanation is that mutation resulted in the gain of ability to produce a single permease required for the induction of

both enzymes. Under this hypothesis, it would be difficult to understand how a strain could have become wild-type during evolution when it could not utilize two enzymes it was capable of synthesizing. Kline & Baron (126) have reported a similar situation in *S. typhimurium* in which a single mutation results in the ability to synthesize both xylose isomerase and xylulose kinase. It is possible that in both of these cases a single mutation has resulted in damage to a region concerning a number of discrete recombinable sites (a multiple-site mutation) which affect the syntheses of related enzymes. If this were correct, one would predict the finding of two closely-linked mutations, allelic with the multiple-site mutation and recombinable with each other, each affecting the synthesis of a single enzyme in the related pair.

Gots & Gollub (82) have described a more complicated case in certain adenine-requiring mutant strains of *E. coli* and *S. typhimurium*. In these mutants two different enzymatic activities disappear, one involving the deacylation of the ribotide of 5-amino-4-imidazole-N-succinylocarboxamide, the other involving the deacylation of adenylosuccinic acid to yield adenylic acid. The interesting point is that these two biochemical steps, unlike the previous ones mentioned, are at quite different levels in the biosynthesis of adenine. In this case, mutation has resulted in impaired synthesis of either a bifunctional deacylase or of two enzymes which do not catalyze reactions in immediate sequence in a biosynthetic pathway.

De Robichon-Szulmajster (49) has observed what appears to be the induction of several sequentially-related enzymes by the substrate of one of them. The normal pathway of galactose utilization in *Saccharomyces cerevisiae* is the following: galactose + ATP (catalyzed by galactokinase) → Gal-1-P + ADP; Gal-1-P + UDPG (catalyzed by Gal-1-P uridyl transferase) ⩾ UDP Gal + G-1-P; UDP Gal (catalyzed by UDP Gal-4-epimerase) ⩾ UDPG. A mutant incapable of producing galactokinase in the presence or absence of galactose can, nevertheless, be induced to form both Gal-1-P uridyl transferase and UDP Gal-4-epimerase in the presence of galactose.

Finally, strong evidence has been put forward by Kurahashi (127a) showing that the formation of the same enzyme may be affected by unlinked genes. Two mutants of *E. coli* K12, previously shown to be incapable of utilizing galactose and affected at separate, distant loci (159, 160), were both found to be incapable of producing galactokinase, while several closely linked loci were shown to affect the production of different enzymes (Gal-1-P uridyl transferase or UDP Gal-4-epimerase) in the pathway of galactose utilization (which is similar to that in *Saccharomyces*). One of the methods employed for testing enzymatic activity ruled out the possibility of inhibitors in the extracts from the mutant strains.

### Interaction of Genes

The flagellar antigens of a given *Salmonella* type occur in two alternative phases. Lederberg & Iino (137) have shown, by transduction analysis, that

each phase corresponds to a distinct locus, $H_1$ and $H_2$. Alternation between phases corresponds to alternative expressions of these two loci. By performing transductions between types, with the donor and recipient in various phases, they were also able to show that expression of the $H_1$ locus depended upon the phase of the recipient, which retained the original $H_2$ locus. Expression of a transduced $H_2$ locus, on the other hand, depended upon the phase of the donor cell. Thus, the antigenic phase appeared to be determined by the $H_2$ locus which, in turn, can exist in one of two stable states. Iino & Lederberg (112) have recently analyzed some monophasic variants in *Salmonella*. These variants arise from mutations at the $H_1$ locus which result in arrested synthesis of $H_1$ flagellar proteins. When the bacterium is in phase 1, no flagella are formed and the bacterium is nonmotile. Such mutations are different from those occurring at another locus, $Fla^+ \rightarrow Fla^-$, which result in suppression of flagella formation in either the $H_1$ or $H_2$ phase.

### Pleiotropic Effects of Gene Mutation

The primary effects of a gene mutation may have multiple secondary effects. Bird & Gots (18) have shown that the tryptophan-requiring, phage T1-resistant mutant of *E. coli* B is rapidly killed by a substance produced by wild-type cells in the presence of penicillin. For this reason, this mutant cannot be selected for its tryptophan requirement by means of the penicillin method. Gross (89) has found that a *Neurospora* mutant produces two enzymes not found in the wild-type grown under the same conditions because of the fact that two substances accumulate as a result of a single blocked reaction. The two accumulating substances induce the formation of enzymes which convert them, and they can do so in wild-type as well as in the mutant. Sistrom, Griffiths & Stanier (85, 197) have obtained from the photosynthetic bacterium *Rhodopseudomonas spheroides* some mutant derivatives characterized by impaired synthesis of carotenoid pigments. As a result of this condition, the mutants are deprived of a protective action of the carotenoids against photosensitization of bacteriochlorophyll in the presence of air and light (84).

Englesberg & Ingraham (56) have examined the basis of the amino acid requirements of the fastidious heterotroph *Pasteurella pestis*. The amino acids are needed, not because they cannot be synthesized, but in order to overcome inhibition by other amino acids. The particular requirements are part of a balanced and complex relationship. Neidhardt & Magasanik (162, 163) found that glucose inhibits histidase synthesis in *Aerobacter aerogenes*. The glucose inhibition disappears when L-histidine is substituted for ammonium sulfate in the culture medium of wild-type or when L-histidine is substituted for glutamic acid in the culture medium of a glutamic acid-requiring mutant. Complicated feedback mechanisms are visualized that control the synthesis of catabolic enzymes by the levels of intermediary metabolites.

## EVOLUTION OF GENETIC MATERIAL

The definition of a bacterial species has always been a difficult matter, because there are numerous instances of "strains" (species?) intermediate between two species possessing fairly stable constellations of biochemical, ecological, and morphological characteristics (15), as well as numerous instances of mutations affecting characters which serve to discriminate between species (3, 180). With the advance in our knowledge of genetic recombination in bacteria, it may be possible to borrow a criterion which has been used to distinguish between species of higher organisms. Whenever any mechanism has developed which effectively prevents genetic exchange between populations, they have become isolated and may be considered different species (50). The mechanisms bringing about such genetic isolation in bacteria are not difficult to imagine. If transformation is the means of genetic transfer in a given population, then, when the adsorbing sites on the bacterial surface become so altered through mutation as to exclude certain DNA molecules, an isolating mechanism has arisen. If transduction is the mode of genetic transfer, then mutations in a bacterium furnishing it immunity to infection by certain viruses, or mutations in the viruses altering their host range, are means of limiting genetic exchange between bacterial populations. Where conjugation is the mode of transfer, the F+ agent may undergo such differentiation as to exclude chromosome transfer from a donor bacterium to a recipient. Finally, when the genetic material of a bacterium, which we may assume to be DNA, has become so differentiated through mutation and recombination that it ceases to be compatible with (presumably incapable of pairing and recombining with) the DNA of another bacterium of similar origin, we may recognize the bacteria as belonging to different species.

Bacterial populations may also become ecologically differentiated as a result of nutritional and physiological requirements built up in the course of fitting an ecological niche, as in the case of a parasitic departure from a free-living group of organisms. In this event, the probability of gene transfer between the two populations is exceedingly slight, although no barrier such as those described above may have been developed.

The advantage of isolating mechanisms of this kind, of course, is that they limit the amount of genetic variability in a population which has become well-adapted to a particular ecological niche, thanks to the gamut of genotypes which have been selected over others arising in the course of evolution. Variability is advantageous up to the point where it threatens to break down the probability of adaptive correspondence between the prevailing genotypes and the exigencies of the environment. Genetic isolation divides the vast pool of variability present in the entire bacterial universe into smaller, separated lots.

Experimental investigation of the ideas just discussed is already under way. Inter-"specific" transformation has been described (19, 139, 190), and,

in general, is less efficient than intra-"specific" transformation. The lower efficiency is probably not the result of any difficulty in taking up heterologous DNA, but in incorporating it genetically (193). Heterokaryosis between species of *Streptomyces* has not been found, although it does occur within a given species (21). Differentiation of the F+ agent is also known to occur (16, 74, 182). A particularly interesting study has been that of Luria & Burrous (152) on conjugation and consequent hybridization between strains of *E. coli* and strains of *Shigella*. A number of *Shigella* strains tested were found to behave like F− with F+ or Hfr strains of *E. coli* K12 or C. They were converted to the F+ state by mixed cultivation with an F+ culture of *E. coli*. Hybrids were also produced which possessed combinations of characters typical of *Shigella* with characters typical of *E. coli*. The frequency of conjugation, as determined by zygotic induction of prophage λ, was as high between *Shigella* and *Escherichia* as between F+ and F− strains of *E. coli*. Nevertheless, the frequency of recombination was lower than in similar crosses between strains of *E. coli*. Furthermore, some characters of the *E. coli* parent failed to be transferred to the hybrids, which suggests an incomplete genetic homology between *Shigella* and *Escherichia*. These results point the way to further investigations of the genetic basis of bacterial evolution and to a rational system of bacterial taxonomy based on knowledge of the degrees of genetic homology between organisms.

## LITERATURE CITED

1. Anderson, T. F., and Mazé, R., *Ann. inst. Pasteur,* **93,** 194 (1957)
2. Anderson, T. F., Wollman, E. L., and Jacob, F., *Ann. inst. Pasteur,* **93,** 450 (1957)
3. Barksdale, W. L., Li, K., Cummins, C. S., and Harris, H., *J. Gen. Microbiol.,* **16,** 749 (1957)
4. Barner, H. D., and Cohen, S. S., *J. Bacteriol.,* **68,** 80 (1954)
5. Barner, H. D., and Cohen, S. S., *J. Bacteriol.,* **71,** 149–57 (1956)
6. Barner, H. D., and Cohen, S. S., *J. Bacteriol.,* **74,** 350–55 (1957)
7. Baron, L. S., Formal, S. B., and Washington, O., *Virology,* **3,** 417 (1957)
8. Beale, G., *The Genetics of Paramecium aurelia* (Cambridge Monograph Exptl. Biol. No. 2, Cambridge University Press, London, Engl., 1954)
9. Bećarević, A., *Biochim. et Biophys. Acta,* **25,** 161 (1957)
10. Bećarević, A., *Biochim et Biophys. Acta,* **25,** 299 (1957)
11. Bećarević, A., *Biochim. et Biophys. Acta,* **25,** 646 (1957)
12. Belser, W. L., and Bunting, M. I., *J. Bacteriol.,* **72,** 582 (1956)
13. Bendich, A., Pahl, H. B., and Beiser, S. M., *Cold Spring Harbor Symposia Quant. Biol.,* **21,** 31 (1956)
14. Benzer, S., *The Chemical Basis of Heredity,* 70–93 (Johns Hopkins Press, Baltimore, Md., 848 pp., 1957)
15. Bergey's *Manual of Determinative Bacteriology,* 7th ed. (The Williams & Wilkins Co., Baltimore, Md., 1900)
16. Bernstein, H., *Heredity,* **11,** 154 (1957)
17. Bertani, G., *Advances in Virus Research,* **5** (1957), (Preprint)

18. Bird, T. J., and Gots, J. S., *J. Bacteriol.*, **71**, 673 (1956)
19. Bracco, R. M., Krauss, M. R., Roe, A. S., and MacLeod, C. M., *J. Exptl. Med.*, **106**, 247–59 (1957)
20. Bradley, S. G., *J. Bacteriol.*, **73**, 581 (1957)
21. Braendle, D. H., and Szybalski, W., *Proc. Natl. Acad. Sci. U.S.*, **43**, 947 (1957)
22. Brenner, S., *Proc. Natl. Acad. Sci. U.S.*, **43**, 687 (1957)
23. Brown, E. R., Cherry, W. B., Moody, M. D., and Gordon, M. A., *J. Bacteriol.*, **69**, 590 (1955)
24. Brown, G. L., and Ephrussi-Taylor, H., in Ephrussi-Taylor, H., *Advances in Virus Research*, **3**, 275 (1955)
25. Brown, G. L., and Martin, A. V., *Nature*, **176**, 971 (1956)
26. Butler, J. A. V., Phillips, D. M., and Shooter, K. V., *Arch. Biochem. Biophys.*, **71**, 423 (1957)
27. Campbell, A., *Virology*, **4**, 366 (1957)
28. Campbell, A., *Bacteriol. Rev.*, **21**, 263–72 (1957)
29. Carey, W. F., Spilman, W., and Baron, L. S., *J. Bacteriol.*, **74**, 543 (1957)
30. Cavalieri, L. F., *J. Am. Chem. Soc.*, **79**, 5319 (1957)
31. Cavalli-Sforza, L. L., *Boll. ist. sieroterap. milan*, **29**, 1 (1950)
32. Cavalli-Sforza, L. L., *Ann. Rev. Microbiol.*, **11**, 391–418 (1957)
33. Cavalli-Sforza, L. L., and Jinks, L. L., *J. Genet.*, **54**, 87 (1956)
34. Cavalli-Sforza, L. L., Lederberg, J., and Lederberg, E. M., *J. Gen. Microbiol.*, **8**, 89 (1953)
35. Chargaff, E., Schulman, H. M., and Shapiro, H. S., *Nature*, **180**, 851 (1957)
36. Clowes, R. C., *J. Gen. Microbiol.*, **17**, xvii (1957)
37. Cohen, S. S., and Barner, H. D., *Proc. Natl. Acad. Sci. U.S.*, **40**, 885–93 (1954)
38. Cohen, S. S., and Barner, H. D., *J. Bacteriol.*, **71**, 588–97 (1956)
39. Corey, R. R., and Starr, M. P., *J. Bacteriol.*, **74**, 137–40 (1957)
40. Corey, R. R., and Starr, M. P., *J. Bacteriol*, **74**, 141–45 (1957)
41. Corey, R. R., and Starr, M. P., *J. Bacteriol.*, **74**, 146–50 (1957)
42. Coughlin, C. A., and Adelberg, E. A., *Nature*, **178**, 529–32 (1956)
43. Crick, F. H. C., Griffith, J. S., and Orgel, L. E., *Proc. Natl. Acad. Sci. U.S.*, **43**, 416 (1957)
44. Delbrück, M., and Stent, G., *Chemical Basis of Heredity*, 699 (Johns Hopkins Press, Baltimore, Md., 848 pp., 1957)
45. Demerec, M., *Symposia Soc. Exptl. Biol.*, No. **7**, 43–54 (1953)
46. Demerec, M., *Cold Spring Harbor Symposia Quant. Biol.*, **21**, 113 (1956)
47. Demerec, M., and Demerec, Z. E., *Brookhaven Symposia in Biol., No. 8* (BNL 350 (C-22)), 75, (1956)
48. Demerec, M., and Hartman, Z., *Carnegie Inst. Wash. Publ. 612*, 5 (1956)
49. de Robichon-Szulmajster, H., *Science*, **127**, 28 (1958)
50. Dobzhansky, T., *Genetics and the Origin of Species*, 3rd ed. (Columbia Univ. Press, New York, N.Y., 364 pp., 1951)
51. Durham, N. N., and Wyss, O. *J. Bacteriol.*, **74**, 548–52 (1957)
52. Englesberg, E., *J. Bacteriol.*, **73**, 641–48 (1957)
53. Englesberg, E., *Arch. Biochem. Biophys.*, **71**, 179 (1957)
54. Englesberg, E., *J. Bacteriol.*, **74**, 8 (1957)
55. Englesberg, E., *Records Genet. Soc. Am.*, **26**, 368 (1957)
56. Englesberg, E., and Ingraham, L., *Proc. Natl. Acad. Sci. U.S.*, **43**, 369 (1957)
57. Ephrussi, B., *Harvey Lectures, Ser. 46*, 45 (1952)

57a. Ephrussi, B., *Nucleocytoplasmic Relations in Microorganisms* (Clarendon Press, Oxford, Engl., 127 pp., 1953)
58. Ephrussi-Taylor, H., and Latarjet, R., *Biochim. et Biophys. Acta*, **16**, 183 (1955)
59. Ephrussi-Taylor, H., *Chemical Basis of Heredity*, 299–320 (Johns Hopkins Press, Baltimore, Md., 848 pp., 1957)
60. Falcone, G., and Szybalski, W., *Exptl. Cell Research*, **11**, 486–89 (1956)
61. Fenn, W. O., Gerschman, R., Gilbert, D. L., Terwilliger, D. E., and Cothran, F. V., *Proc. Natl. Acad. Sci. U.S.*, **43**, 1027–32 (1957)
62. Fincham, J. R. S., and Boylen, J. B., *J. Gen. Microbiol.*, **16**, 438 (1957)
63. Fincham, J. R. S., and Pateman, J. A., *Heredity*, **11**, 285 (1957)
64. Fischer-Fantuzzi, L., Rita, G., and Russi, M., *Nuovi ann. igiene e microbiol.*, **7**, 1 (1956)
65. Fisher, K. W., *J. Gen. Microbiol.*, **16**, 120 (1957)
66. Fisher, K. W., *J. Gen. Microbiol.*, **16**, 136 (1957)
67. Forro, F., Jr., *Exptl. Cell Research*, **12**, 363–73 (1957)
68. Fox, M. S., *J. Gen. Physiol.*, **39**, 267–78 (1955)
69. Fox, M. S., *Biochim. et Biophys. Acta*, **26**, 83–85 (1957)
70. Fox, M. S., and Hotchkiss, R. D., *Nature*, **179**, 1322 (1957)
71. Fraser, D., Mahler, H. R., Shug, A. L., and Thomas, C. A., Jr., *Proc. Natl. Acad. Sci. U.S.*, **43**, 939 (1957)
72. Feurst, C. R., and Stent, G. S., *J. Gen. Physiol.*, **40**, 73 (1956)
73. Fuerst, C. R., Jacob, F., and Wollman, E. L., *Compt. rend.*, **243**, 2162 (1956)
74. Furness, G., and Rowley, D., *J. Gen. Microbiol.*, **17**, 550 (1957)
75. Garen, A., and Zinder, N. D., *Virology*, **1**, 347 (1955)
76. Giles, N. H., Jr., *Cold Spring Harbor Symposia Quant. Biol.*, **16**, 283 (1951)
77. Giles, N. H., Jr., Partridge, C. W. H., and Nelson, N. J., *Proc. Natl. Acad. Sci. U. S.*, **43**, 305–17 (1957)
78. Glover, S. W., *Carnegie Inst. Wash. Publ.* 612, 121 (1956)
79. Goodgal, S. H., and Herriott, R. M., *Records Genet. Soc. Am.*, **26**, 372 (1957)
80. Goodgal, S. H., and Herriott, R. M., *Chemical Basis of Heredity*, 336–40 (Johns Hopkins Press, Baltimore, Md., 848 pp., 1957)
81. Goodgal, S. H., Rupert, C. S., and Herriott, R. M., *Chemical Basis of Heredity*, 341–43 (Johns Hopkins Press, Baltimore, Md., 848 pp., 1957)
82. Gots, J. S., and Gollub, E. G., *Proc. Natl. Acad. Sci. U.S.*, **43**, 826 (1957)
83. Green, J. A., and Williams, R. P., *J. Bacteriol.*, **74**, 633 (1957)
84. Griffiths, M., Sistrom, W. R., Cohen-Bazire, G., and Stanier, R. Y., *Nature*, **176**, 1211 (1955)
85. Griffiths, M., and Stanier, R. Y., *J. Gen. Microbiol.*, **14**, 698 (1956)
86. Groman, N. B., *J. Bacteriol.*, **69**, 9 (1955)
87. Groman, N. B., *J. Bacteriol.*, **70**, 637 (1955)
88. Groman, N. B., *Virology*, **2**, 843 (1956)
89. Gross, S. R., *Records Genet. Soc. Am.*, **26**, 374 (1957)
90. Guild, W. R., and DeFilippes, F. M., *Biochim. et Biophys. Acta*, **26**, 241 (1957)
91. Haas, F. L., and Doudney, C. O., *Proc. Natl. Acad. Sci. U.S.*, **43**, 871–83 (1957)
92. Haas, F. L., and Doudney, C. O., *Records Genet. Soc. Am.*, **26**, 374 (1957)
93. Hanson, E. D., *Genetics*, **41**, 21–30 (1956)
94. Hartman, P. E., *Carnegie Inst. Wash. Publ.* 612, 35 (1956)
95. Hartman, P. E., *Chemical Basis of Heredity*, 408 (Johns Hopkins Press, Baltimore, Md., 848 pp., 1957)

96. Hartman, Z., *Carnegie Inst. Wash. Publ. 612,* 107 (1956)
97. Hashimoto, K., *Japan. J. Microbiol.,* **1,** 1 (1957)
98. Hayes, W., *Cold Spring Harbor Symposia Quant. Biol.,* **18,** 75 (1953)
99. Hayes, W., *J. Gen. Microbiol.,* **16,** 97 (1957)
100. Hirota, Y., *Nature,* **178,** 92 (1956)
101. Holman, H. R., Robbins, W. C., Deicher, H., and Kunkel, H. G., *Science* **126,** 1232 (1957)
102. Horowitz, N. H., and Fling, M., *Proc. Natl. Acad. Sci. U.S.,* **42,** 498 (1956)
103. Hotchkiss, R. D., *Cold Spring Harbor Symposia Quant. Biol.,* **16,** 457 (1951)
104. Hotchkiss, R. D., *Phosphorus Metabolism,* 2 (Johns Hopkins Press, Baltimore, Md., 946 pp., 1952)
105. Hotchkiss, R. D., *Harvey Lectures, Ser. 49,* 124 (1954)
106. Hotchkiss, R. D., *Proc. Natl. Acad. Sci. U.S.,* **40,** 49 (1954)
107. Hotchkiss, R. D., *The Nucleic Acids,* 2 (Academic Press, Inc., New York, N.Y., 576 pp., 1955)
108. Hotchkiss, R. D., *Enzymes: Units of Biological Structure and Function* (Academic Press, Inc., New York, N.Y., 646 pp., 1956)
109. Hotchkiss, R. D., *Chemical Basis of Heredity,* 321 (Johns Hopkins Press, Baltimore, Md., 848 pp., 1957)
110. Hotchkiss, R. D., and Marmur, J., *Proc. Natl. Acad. Sci. U.S.,* **40,** 55 (1954)
111. Hotchkiss, R. D., and Evans, A. H., *Science,* **126,** 1232 (1957)
112. Iino, T., and Lederberg, J., *Records Genet. Soc. Am.,* **26,** 378 (1957)
113. Iverson, R. M., and Giese, A. M., *Biochim. et Biophys. Acta,* **25,** 62–68 (1957)
114. Iverson, R. M., and Giese, A. M., *Exptl. Cell Research,* **13,** 213–23 (1957)
115. Jacob, F., and Wollman, E. L., *Ann. inst. Pasteur,* **91,** 486 (1956)
116. Jacob, F., and Wollman, E. L., *Compt. rend.,* **242,** 303 (1956)
117. Jacob, F., and Wollman, E. L., *Chemical Basis of Heredity,* 468 (Johns Hopkins Press, Baltimore, Md., 848 pp., 1957)
118. Jacob, F., and Wollman, E. L., *Symposia Soc. Exptl. Biol., No. 12* (1957)
119. Jagger, J., and Latarjet, R., *Ann. inst. Pasteur,* **91,** 858-73 (1956)
120. Jensen, K. A., Kirk, I., Kolmark, G., and Westergaard, M., *Cold Spring Harbor Symposia Quant. Biol.,* **16,** 245 (1951)
121. Kanazir, D., in Witkin, E. M., *Cold Spring Harbor Symposia Quant. Biol.,* **21,** 123 (1956)
122. Kanazir, D., and Errera, M., *Cold Spring Harbor Symposia Quant. Biol.,* **21,** 19–30 (1956)
123. Kelner, A., *J. Gen. Physiol.,* **34,** 385 (1951)
124. Kimball, R. F., Gaither, N., and Wilson, S. M., *Genetics,* **42,** 661–69 (1957)
125. Kirchner, C., and Eisenstark, A., *Records Genet. Soc. Am.,* **26,** 381 (1957)
126. Kline, E. S., and Baron, L. S., *Arch. Biochem. Biophys.,* **66,** 128 (1957)
127. Kølmark, G., *Compt. rend. trav. lab. Carlsberg, Sér. physiol.,* **26,** 205–20 (1956)
127a. Kurahashi, K., *Science,* **125,** 114–16 (1957)
128. Lark, K. G., and Maaløe, O., *Biochim. et Biophys. Acta,* **21,** 448–58 (1956)
129. Latarjet, R., and Beljanski, M., *Ann. inst. Pasteur,* **90,** 127–32 (1956)
130. Lederberg, E. M., and Lederberg, J., *Genetics,* **38,** 51 (1953)
131. Lederberg, J., *Genetics,* **32,** 505–25 (1947)
132. Lederberg, J., *J. Cellular Comp. Physiol.,* **45,** Suppl. 2, 75 (1955)
133. Lederberg, J., *Proc. Natl. Acad. Sci. U.S.,* **42,** 574 (1956)
133a. Lederberg, J., *Genetics,* **41,** 845 (1956)
134. Lederberg, J., *Bacteriol. Rev.,* **21,** 133–39 (1957)

135. Lederberg, J., *J. Bacteriol.*, **73**, 144 (1957)
136. Lederberg, J., *Proc. Natl. Acad. Sci. U.S.*, **43**, 1060 (1957)
137. Lederberg, J., and Iino, T., *Genetics*, **41**, 743 (1956)
138. Lederberg, J., and Lederberg, E., *Cellular Mechanisms in Differentiation and Growth* (Princeton Univ. Press, Princeton, N.J., 244 pp., 1956)
139. Leidy, G., Hahn, E., and Alexander, H. E., *J. Exptl. Med.*, **104**, 305–20 (1956)
140. Le Minor, L., and Le Minor, S., quoted in Wollman, E. L., Jacob, F., and Hayes, W., *Cold Spring Harbor Symposia Quant. Biol.*, **21** (1956)
141. Lerman, L. S., *Biochim. et Biophys. Acta*, **18**, 132 (1955)
142. Lerman, L. S., *Cold Spring Harbor Symposia Quant. Biol.*, **21** (Discussion following Bendich *et al.*, 1956)
143. Lerman, L. S., and Tolmach, L. J., *Biochim. et Biophys. Acta*, **26**, 68–82 (1957)
144. Lerner, P., and Yanofsky, C., *J. Bacteriol.*, **74**, 494 (1957)
145. Lester, G., and Bonner, D. M., *J. Bacteriol.*, **73**, 544 (1957)
146. Levine, M., *Virology*, **3**, 22 (1957)
147. Levinthal, C., *Proc. Natl. Acad. Sci. U.S.*, **42**, 394–404 (1956)
148. Levinthal, C., and Thomas, C. A., Jr., *Biochim. et Biophys. Acta*, **23**, 453 (1957)
149. Lewis, G. B., *Records Genet. Soc. Am.*, **26**, 383 (1957)
150. Lieb, M., *Ann. inst. Pasteur*, **93**, 52 (1957)
151. Litman, R. M., and Pardee, A. B., *Nature*, **178**, 529–32 (1956)
152. Luria, S. E., and Burrous, J. W., *J. Bacteriol.*, **74**, 461 (1957)
153. Luria, S. E., and Human, M. L., *J. Bacteriol.*, **64**, 557 (1952)
154. Lwoff, A., *Bacteriol Rev.*, **17**, 269 (1953)
155. Maccacaro, G. A., and Comolli, R., *J. Gen. Microbiol.*, **15**, 121 (1956)
156. MacLeod, C. M., and Krauss, M., *J. Exptl. Med.*, **103**, 623–32 (1956)
157. Marcovich, H., *Ann. inst. Pasteur*, 93, 456–62 (1957)
157a. Meselson, M., and Stahl, F. W., *Proc. Biophys. Soc.*, Paper A4 (1958)
157b. Meselson, M., Stahl, F. W., and Vinograd, J., *Proc. Acad. Sci. U.S.*, **43**, 581 (1957)
158. Morse, M. L., *Ann. N. Y. Acad. Sci.*, **68**, 324 (1957)
159. Morse, M. L., Lederberg, E. M., and Lederberg, J., *Genetics*, **41**, 142 (1956)
160. Morse, M. L., Lederberg, E. M., and Lederberg, J., *Genetics*, **41**, 758 (1956)
161. Moser, H., *Yearbook, Carnegie Inst. Wash.*, 237 (1954)
162. Neidhardt, F. C., and Magasanik, B., *J. Bacteriol.*, **73**, 253 (1957)
163. Neidhardt, F. C., and Magasanik, B., *J. Bacteriol.*, **73**, 260 (1957)
164. Nelson, T. C., and Lederberg, J., *Proc. Natl. Acad. Sci. U.S.*, **40**, 415 (1954)
165. Newmeyer, D., *J. Gen. Microbiol.*, **16**, 449 (1957)
166. Novick, A., *Ann. Rev. Microbiol.*, **9**, 97–110 (1955)
167. Novick, A., and Szilard, L., *Cold Spring Harbor Symposia Quant. Biol.*, **16**, 337–43 (1951)
168. Novick, A., and Weiner, M., *Proc. Natl. Acad. Sci. U.S.*, **43**, 553–66 (1957)
169. Ogg, J. E., and Zelle, M. R., *J. Bacteriol.*, **74**, 477–84 (1957)
170. Ozeki, H., *Carnegie Inst. Wash. Publ. 612*, 97 (1956)
171. Painter, R. B., Forro, F., Jr., and Hughes, W. L., Jr., *Bacteriol. Proc.* (*Soc. Am. Bacteriologists*), (1957)
172. Pateman, J. A., and Fincham, J. R. S., *Heredity*, **11**, 285 (1957)
173. Phillips, J. H., Braun, W., and Plescia, O., *Records Genet. Soc. Am.*, **26**, 389 (1957)

174. Plaut, W., and Mazia, D., *J. Biophys. Biochem. Cytol.*, **2**, 573-88 (1956)
175. Prescott, D. M., *Exptl. Cell Research*, **12**, 196 (1957)
176. Prestidge, L. S., and Pardee, A. B., *J. Bacteriol.*, **74**, 48 (1957)
177. Provasoli, L., Hutner, S. H., and Schatz, A., *Proc. Soc. Exptl. Biol. Med.*, **69**, 279 (1948)
178. Quadling, C., and Stocker, B. A. D., *J. Gen. Microbiol.*, **17**, 424–36 (1957)
179. Quadling, C., and Stocker, B. A. D., *J. Gen. Microbiol.*, **14**, i (1957)
180. Ravin, A. W., *J. Gen. Microbiol.*, **6**, 211 (1952)
181. Ravin, A. W., *Ann. N.Y. Acad. Sci.*, **68**, 335–48 (1957)
182. Richter, A., *Records Genet. Soc. Am.*, **26**, 391 (1957)
183. Rickenberg, H. V., *Biochim. et Biophys. Acta*, **25**, 206 (1957)
184. Robinow, C. F., Addendum to Dubos, R., *The Bacterial Cell* (Harvard Univ. Press, Cambridge, Mass., 460 pp., 1947)
185. Romig, W. R., Socolofsky, M. D., and Wyss, O., *Bacteriol. Proc. (Soc. Am. Bacteriologists)*, Paper No. G54 (1956)
186. Ryan, F. J., *Genetics*, **40**, 726–38 (1955)
187. Ryan, F. J., *Ann. N. Y. Acad. Sci.* (In press, 1957)
188. Ryan, F. J., and Kiritani, K., *J. Bacteriol.* (In press, 1958)
189. Santer, U. V., and Vogel, H. J., *Biochim. et Biophys. Acta*, **19**, 578 (1956)
190. Schaeffer, P., *Ann. inst. Pasteur*, **91**, 192–211 (1956)
191. Schaeffer, P., *Ann. inst. Pasteur*, **91**, 323–37 (1956)
192. Schaeffer, P., *Compt. rend.*, **245**, 230–31 (1957)
193. Schaeffer, P., *Compt. rend.*, **245**, 375–77 (1957)
194. Schaeffer, P., *Compt. rend.*, **245**, 451–53 (1957)
195. Scott, D. B. M., and Chu, E., *Exptl. Cell Research*, **14**, 166–74 (1958)
196. Sermonti, G., and Spada-Sermonti, I., *J. Gen. Microbiol.*, **15**, 609 (1956)
197. Sistrom, W. R., Griffiths, M., and Stanier, R. Y., *J. Cellular Comp. Physiol.*, **48**, 473 (1956)
198. Skaar, P. D., *Records Genet. Soc. Am.*, **26**, 395 (1957)
199. Skaar, P. D., and Garen, A., *Proc. Natl. Acad. Sci. U.S.*, **42**, 619 (1956)
200. Skaar, P. D., Richter, A., and Lederberg, J., *Proc. Natl. Acad. Sci. U.S.*, **43**, 329 (1957)
201. Sonneborn, T. M., *Heredity*, **4**, 11 (1950)
202. Spärck, J. V., *Compt. rend. trav. lab. Carlsberg, Sér. physiol.*, **26**, 349–61 (1956)
203. Spiegelman, S., *Enzymes: Units of Biological Structure and Function* (Academic Press, Inc., New York, N.Y., 646 pp., 1956)
204. Spiegelman, S., *Chemical Basis of Heredity*, 232 (Johns Hopkins Press, Baltimore, Md., 848 pp., 1957)
205. Spizizen, J., *Proc. Natl. Acad. Sci. U.S.*, **43**, 694 (1957)
206. Starlinger, P., and Kaudewitz, F., *Z. Naturforsch.*, **11b**, 317 (1956)
207. Sterne, M., and Proom, H., *J. Bacteriol.*, **74**, 541–42 (1957)
208. Stich, H. F., and Kitiyakara, A., *Science*, **126**, 1019 (1957)
209. Stocker, B. A. D., *J. Gen. Microbiol.*, **15**, 575–98 (1956)
210. Suskind, S. R., *J. Bacteriol.*, **74**, 308 (1957)
211. Suskind, S. R., and Kurek, L. I., *Science*, **126**, 1068 (1957)
212. Swift, H. H., *Intern. Rev. Cytol.*, **2**, 1 (1953)
213. Taylor, H. E., *Compt. rend.*, **228**, 1258 (1949)
214. Taylor, J. H., Woods, P. S., and Hughes, W. L., *Proc. Natl. Acad. Sci. U.S.*, **43**, 122–28 (1957)

215. Thomas, R., *Biochim. et Biophys. Acta,* **18,** 464 (1955)
216. Tolmach, L. J., and Lerman, L. S., *Radiation Research,* **7,** 128 (1957)
217. Uetake, H., Nakagawa, T., and Akiba, T., *J. Bacteriol.,* **69,** 571 (1955)
218. Vogel, H. J., *Proc. Natl. Acad. Sci. U.S.,* **43,** 491 (1957)
219. Vogel, H. J., *Chemical Basis of Heredity,* 276 (Johns Hopkins Press, Baltimore, Md., 848 pp., 1957)
219a. Wainwright, L. K., *J. Gen. Microbiol.,* **14,** 533 (1956)
220. Weigle, J. J., *Proc. Natl. Acad. Sci. U.S.,* **39,** 628 (1953)
221. Weigle, J. J., *Virology,* **4,** 14 (1957)
222. Westergaard, M., *Experientia,* **13,** 224–34 (1957)
223. Witkin, E. M., *Cold Spring Harbor Symposia Quant. Biol.,* **16,** 357–72 (1951)
224. Witkin, E. M., *Cold Spring Harbor Symposia Quant. Biol.,* **21,** 123–40 (1956)
225. Wolff, S., and Atwood, K. C., *Proc. Natl. Acad. Sci. U.S.,* **40,** 187–92 (1954)
226. Wolff, S., and Luippold, H. E., *Science,* **122,** 231–32 (1955)
227. Wollman, E. L., and Jacob, F., *Ann. inst. Pasteur,* **93,** 323 (1957)
227a. Wollman, E. L., Jacob, F., and Hayes, W., *Cold Spring Harbor Quant. Biol.,* **21,** 141 (1956)
228. Woodward, V. W., *Proc. Natl. Acad. Sci. U.S.,* **42,** 752–58 (1956)
229. Yura, T., *Carnegie Inst. Wash. Publ.* 612, 63 (1956)
230. Zamenhof, S., De Giovanni, R., and Rich, K., *J. Bacteriol.,* **71,** 60–69 (1956)
231. Zamenhof, S., De Giovanni, R., and Green, S., *Records. Genet. Soc. Am.,* **26,** 403 (1957)
232. Zamenhof, S., Leidy, G., Greer, S., and Hahn, E., *J. Bacteriol.,* **74,** 194 (1957)
233. Zamenhof, S., Reiner, B., De Giovanni, R., and Rich, K., *J. Biol. Chem.,* **219,** 165–73 (1956)
234. Zelle, M. R., and Ogg, J. E., *J. Bacteriol.,* **74,** 485 (1957)
235. Zinder, N. D., *Cold Spring Harbor Symposia Quant. Biol.,* **18,** 261 (1953)
235a. Zinder, N. D., *Science,* **126,** 1237 (1957)
236. Zinder, N. D., and Arndt, W. F., *Proc. Natl. Acad. Sci. U.S.,* **42,** 586 (1957)
237. Zinder, N. D., and Lederberg, J., *J. Bacteriol.,* **64,** 679 (1952)

# GENETICS OF FUNGI[1]

By Harry E. Wheeler
*Louisiana State University, Baton Rouge, Louisiana*

Since the editor's decision to subdivide the subject of genetics of microorganisms following the review of Zelle (1), an excellent general review of fungal genetics by Emerson (2) and a review of one special area, parasexuality, by Pontecorvo (3) have appeared. A survey of subsequent literature has revealed more than 500 papers dealing wholly or in part with the subject of this review; of these, 159 have been selected for citation. The problem of selection has been somewhat simplified by the appearance of a number of recent reviews of subjects, such as physiological genetics, in which contributions made with the fungi have been adequately covered. Furthermore, several investigators have published reviews of extensive work with a single organism or group of organisms and, in such cases, only the review has been cited in the belief that persons interested in the original papers can readily find the references by consulting the literature cited in the appropriate review. The basis for further selection has been entirely arbitrary and the subjects selected for inclusion probably reflect the interests of the author. However, an attempt has been made to present a fairly complete over-all picture with special attention to areas which have been either overlooked or considered outside the scope of previous reviews.

## Nature, Structure, and Function of Genetic Material

Any critical evaluation of current ideas regarding the nature, structure, and function of genetic material must consider not only genetic evidence from a wide variey of organisms but also chemical and physical data provided by disciplines quite unfamiliar to most geneticists. Several recent books and reviews (4 to 9), as well as two volumes based on papers presented in symposia have dealt with these problems. One of the latter, representing perhaps the most ambitious attempt to date to define genetic material in chemical terms, is made especially valuable to the nonspecialist by an introductory survey by Beadle (10) and a critical and comprehensive summary by Glass (11). Although this review is concerned only with evidence derived from fungi, a brief summary of current concepts seems in order. Most workers now agree that, except for some plant viruses, the fundamental genetic material is primarily, if not entirely, deoxyribonucleic acid (DNA), and find the Watson-Crick model the most satisfactory yet proposed for its structure. This model, a double helix of two polynucleotide chains held together by hydrogen bonds between complementary pairs of bases, serves as the basis for current concepts of the nature and function of

[1] The survey of the literature pertaining to this review was completed in December, 1957.

genes. Genes are conceived as localized units of DNA which are duplicated by replication of the DNA helix and which undergo mutation as the result of structural alterations in the DNA molecule. Genetic information is coded in specific base-pair sequences and is translated into protein specificity perhaps by means of an RNA template.

Although the scheme outlined above represents the majority viewpoint, it is by no means universally accepted. Some workers continue to ascribe an important genetic role to the proteins associated with DNA. Several modifications of the Watson-Crick model have been proposed in attempts to overcome certain of its inherent difficulties. One of the most serious of these difficulties, the problem of separation of the strands of the helix during DNA replication, has received considerable attention (12, 13). Many uncertainties regarding the nature, structure, and function of genetic units remain unresolved and, in some cases, new problems have been created by recent findings. Results of work with fungi having an important bearing on these problems are discussed in the following sections.

*Gene conversion.*—This term, originally coined by Winkler, was resurrected in 1949 by Lindegren who defines it (14) as "the interaction between alleles (A and a) in a heterozygote (A a) that results in either the loss of capacity of a normal allele (A → a) or the restitution of capacity in a defective allele (a → A)."

Between 1949 and 1955, Lindegren and his associates reported several cases of irregular segregation in yeast tetrads which they attributed to gene conversion. This interpretation was vigorously challenged by other investigators working with yeast who pointed out that many other mechanisms could account for the observed irregularities. Several such mechanisms, including extra mitotic divisions in the ascus, the formation of binucleate ascospores, polyploidy, mutation at the mating-type locus and complementary genes, have been shown to be operative in yeast [Winge (15); Roman *et al.* (16); Leupold (17); Roman (18); Saito & Takahashi (19)]. Lindegren and his co-workers in a series of papers (20 to 23), have attempted to answer these criticisms but new objections have been raised and a lively controversy continues (24, 25, 26). The details of this dispute will be omitted since studies with another organism have provided evidence of gene conversion unclouded by the ambiguities surrounding yeast genetics.

Serious consideration of gene conversion as a mutation-like process leading to recombination in the absence of conventional crossing-over dates from Mitchell's analysis of the pyridoxineless mutants in *Neurospora* (27). This and later work on three pyrimidineless mutants (28) has been reviewed by Mitchell (29). The clearest evidence was obtained with mutant alleles at the pyridoxine locus. Crosses between two mutants, *pdx* and *pdxp*, yielded 103 pyridoxine-independent progeny among a total of 66,959 random isolates. That these prototrophs did not arise as the result of crossing over was shown by tetrad analysis. Four of 585 asci from this cross yielded pyridoxine-independent progeny but the double mutant expected if crossing over

had occurred between *pdx* and *pdxp* was not found. Since no prototrophs were obtained when each of the two mutants was selfed, ordinary reverse mutations occurring during nuclear divisions in the ascus could not account for the results. Moreover, short heat treatments known to increase mutation rates, had no significant effect on crossing over between nearby outside markers but markedly reduced the frequency of reversions attributable to gene conversion at the pyridoxine locus.

Further evidence of gene conversion has been obtained in several other cases. In *Neurospora,* St. Lawrence (30) has made a detailed analysis of four niacin-requiring mutants at the *q* locus. When selfed, none of these yielded niacin-independent progeny but interallele crosses between different pairs yielded a few prototrophs. Segregation of outside markers indicated that the prototrophic progeny did not result from a crossover within the *q* region. In another case, heterokaryon experiments with 29 adenine mutants at the *ad-3* locus have shown that they can be separated into two physiological groups. Segregations of closely linked markers on either side of the *ad-3* region indicated that in intergroup crosses adenine-independent progeny resulted from crossing over within the region, whereas those obtained from intragroup crosses arose through some other mechanism [de Serres (31)]. Tetrad analysis of an intergroup cross demonstrated the presence of the double mutant in six asci containing an adenine-independent pair of spores (32). A very similar situation has been reported by Case & Giles (33) who analyzed 937 tetrads from a cross of two mutants at the *pan-2* locus. Of 11 aberrant tetrads, two showed segregations indicating that wild-types and double mutants were recovered as reciprocal products of crossovers. The other nine tetrads, which gave either wild-types but no double mutants or 3:1 ratios of mutant types, were attributed to gene conversion.

In studies with yeast other than those already cited, Roman (18), working with adenine mutants, found that heteroallelic diploids (cultures having two *ad* alleles of different mutational origin) produced reversions to adenine-independence whereas homoallelic combinations (those having two alleles of the same origin) produced very few or no revertants. These tests, carried out during mitotic rather than meiotic division, indicated that recombination in the heteroallelic diploids was of the nonreciprocal type and had occurred without crossing over. Since a study of 83 mutations from dominant to recessive at the *ad* loci showed that 73 differed from each other, it was possible by testing the three recessives obtained from asci secregating 1 *Ad*:3 *ad,* to show that ordinary random mutations during meiosis could not account for the irregular ratios. In some cases, suggestive of gene conversion, authors have chosen to interpret their results on other bases (3, 26). It has been repeatedly emphasized (15, 17, 18, 26) that the rare irregular ratios, found in virtually every organism subjected to tetrad analysis, should not be attributed to gene conversion unless other possible mechanisms have been rigorously eliminated.

*Mechanism of gene conversion.*—Although the occurrence of gene con-

version is widely accepted as a fact, there is little agreement as to its significance, the mechanism responsible for it, or even the proper name for the phenomenon. Lindegren (20) assigns priority for the name to Winkler but both Stern (34) and Beadle (10) have criticized such usage and the latter proposed that the term "transmutation" be adopted. Since transmutation has been used as a synonym for mutation, Glass (11) suggests that an entirely new name would be more appropriate and has proposed the term "transreplication." Preferences in regard to terminology among various investigators generally reflect their viewpoints of the probable mechanism involved. This reviewer, following Bonner (35), has used gene conversion as a convenient term without particular significance regarding the mechanism responsible for the phenomenon.

A number of mechanisms to account for gene conversion have been suggested. Lindegren (14, 20) favors the view that an exchange of genic material between alleles is involved. Mitchell (27) proposed a copy-choice scheme in which a chromatid fragment may be reproduced twice or not at all. St. Lawrence (30) has suggested that controlling elements such as those found in maize may be responsible. Formal models have been presented by Roman (18) and by Mitchell (29), and general discussions of the various schemes advanced by Bonner (35) and St. Lawrence & Bonner (36). At present, all of these schemes must be considered to be purely speculative. Since tetrad analysis, required for clear demonstration of gene conversion, is limited to so few organisms and is at best a tedious method, new methods for recognizing this phenomenon are urgently needed [Mitchell (29)]. It might be noted here that a visible ascospore marker, such as those used with *Bombardia* and *Sordaria,* would allow selection of aberrant asci without the time-consuming process of dissection and spore isolation. The fact that Olive (37), in a study of ascospore color mutants in *Sordaria,* found irregular segregations in heterogygous but not in homozygous asci, suggests that gene conversion may be involved. If markers on either side of the color locus could be obtained, this fungus would then provide an excellent tool for a study of the mechanism responsible for gene conversion.

Obviously, no accurate assessment of the significance of gene conversion can be made until more information is available regarding the extent of its occurrence and the mechanisms involved. Present views range from those who feel that complete re-evaluation of classical genetic methods based on crossing over is required (25), to those who ignore its possible implications in making estimates of the size and structure of genetic units in their material (38, 39). Since the best established cases in *Neurospora* indicate that the phenomenon occurs only about once in each hundred asci, the former view does not appear to be justified. An excellent discussion of more moderate viewpoints has been presented by St. Lawrence & Bonner (36). Several investigators have drawn attention to the similarity of gene conversion to nonreciprocal recombination in bacteriophage (10, 11, 40, 41, 42).

***Gene structure and function.***—Geneticists have long been aware that

genes can be defined as units of mutation, recombination, or function. Benzer (43) has recently proposed three new terms, ***muton, recon,*** and ***cistron,*** for these units and attempted to place limits on the size of each. Since Benzer's work with phage is outside the scope of this review, attention will be given to studies of complex loci in various fungi. One of the most extensive of these has been carried out by Bonner and his co-workers, using tryptophan-requiring mutants at the *td* locus in *Neurospora* (44 to 48). Since the results obtained and their implications have been discussed in recent reviews by Bonner (35), Yanofsky (49), and by Suskind (50), only a brief summary will be presented here. Detailed genetic and biochemical studies of 25 *td* mutants, each of independent origin and each lacking in ability to carry out the tryptophan-synthetase reaction (coupling of indole and serine to form tryptophan), showed that all were located within a limited chromosomal region. Although all of these mutants were allelic on the basis of recombination and heterokaryon tests and all, except one temperature-sensitive strain, failed to produce the enzyme tryptophan-synthetase, they were found to differ in their reactions to specific suppressors and in ability to produce a protein closely related serologically to the active enzyme. These findings have led to the conclusion that the genetic material controlling the formation of a specific enzyme, in this case tryptophan-synthetase, represents a functionally complex but genetically indivisible unit (35, 50).

In other cases, functionally related genetic material appears divisible either by crossing over, gene conversion, or both. Studies, described in the preceding section, of the *pdx* (27) and *q* (30) loci in *Neurospora,* indicate that recombination at these loci can occur by gene conversion but not by crossing over. Investigations of two other loci in *Neurospora, ad-3* and *pan-2,* indicate that in these cases both mechanisms may be involved (31, 32, 33). In many cases it has not been possible to determine the mechanism responsible for recombinations occurring within a limited chromosome region which controls a metabolic reaction (28, 51 to 54). Moreover, as Bonner (35) has emphasized, lack of detailed biochemical knowledge concerning the function of the loci involved may be responsible for much of the apparent confusion.

Studies of a number of apparently complex loci in *Aspergillus nidulans* by Pritchard (38), Calef (55, 56), and other workers (57, 58) have been reviewed by Pontecorvo (39). This work, carried out by mitotic analysis for the most part, indicated that all pairs of alleles of independent origin yielded recombinants when tests capable of detecting recombination fractions of $10^{-7}$ were made. The data obtained were utilized for calculations of the number of sites involved and the size of hereditary units at various loci (39, 59). In regard to such estimates, St. Lawrence & Bonner (36) have pointed out that gene conversion, which may be involved, confuses the issue and makes hazardous attempts to equate the frequency of an unknown event to the size of the hereditary unit. The alternative (40), which assumes intense negative interference with multiple crossovers within a very short

region, does not appear satisfactory for data obtained with other organisms (30). The fact that reciprocal recombination products were recovered in a few cases with *Aspergillus* (38, 60), does not furnish conclusive evidence that these arose as the result of a single event, since two occurrences of gene conversion could lead to the same result. Although the latter seems unlikely in view of the low frequency of recombination observed, it must be noted that mitotic analysis, which assumes somatic crossing over, lacks a firm cytological basis in the fungi. Recent reports of nonmitotic nuclear division in the vegetative hyphae of the Mucorales (61, 62) and certain Basidiomycetes (63), though difficult to reconcile with available genetic data, emphasize the urgent need for more detailed cytological information regarding the nature of nuclei in the vegetative stage.

*Genetic control of metabolic processes.*—The great activity in this area is reflected by a number of recent books and reviews devoted to this subject. Volumes by Haldane (64) and by Wagner & Mitchell (4) provide excellent coverage of work up to 1955. Later developments have been discussed in several reviews (5, 6, 7, 9, 10), of which the most comprehensive is that by DeBusk (9). Since a review of work with fungi would merely duplicate much of the material in the references cited above, only a few selected themes will be considered here.

The current status of the "one gene—one enzyme" hypothesis has been discussed by DeBusk (9), Horowitz (65), Horowitz & Fling (66), Lederberg (67), and Yanofsky (49). Proponents of this hypothesis cite the following lines of evidence in its favor: Most mutations show effects which can be accounted for by loss of a single biochemical reaction. In a number of cases, mutants having metabolic blocks also show a deficiency of the corresponding enzyme. Several cases of mutants with double or multiple requirements have been shown to be the result of a block in the synthesis of a common precursor and thus no longer conflict with the hypothesis. Cases of inhibition of amino acid-requiring mutants by specific amino acids, also once suggestive of multifunctional gene action, can now be explained on the basis of work by Mathieson & Catcheside (68) who reported that such inhibition may arise from prevention of uptake of the required nutrilite from the medium. Horowitz & Fling (66) conclude that while the evidence that one gene functions in the synthesis of only one enzyme is reasonably clear, it is quite possible that one enzyme may result from the cooperative action of more than one gene. In regard to the latter, several reports of the formation of an enzyme by complementary gene action have appeared (27, 28, 69, 70, 71).

The template hypothesis of gene function has been discussed by several workers (9, 10, 65, 66, 67) and a lucid exposition of the possible role of templates in protein synthesis has been presented by Spiegelman (72). Horowitz (65) and Horowitz & Fling (66) have pointed out that if the template hypothesis is correct, qualitative as well as quantitative alterations of enzymes would be expected as products of different mutant alleles. Their

extensive studies of tyrosinase thermostability in *Neurospora* provide an example of such qualitative alteration. Although there is general agreement that the one gene—one enzyme hypothesis of gene function remains the most effective yet devised, many share Lederberg's (67) view that it would be premature to insist that present evidence shows that this function is the synthesis of a particular enzyme protein. Moreover, Delbruck's objection, voiced more than a decade ago, still applies (67); namely, no experimental test is available which can exclude the one—one hypothesis short of detailed knowledge of how genes act.

## Tetrad Analysis

At the beginning of the period under review, Perkins (73) published a detailed survey of results obtained with various organisms by means of analysis of tetrads. In considering the use of tetrads for measuring crossing over and estimating map distances, Perkins gave special attention to data from various sources which deviated from expectations based on the assumption that crossing over occurs at random. Two types of deviations were noted: those cases in which tetratype frequencies exceeded theoretical expectations and those in which the numbers of two-, three-, and four-strand double exchanges deviated from the 1:2:1 ratio expected. In regard to the latter, Perkins pointed out that some but not all of the cases attributed by the Lindegrens to chromatid interference could be accounted for by assuming a low frequency of misassortment of centromeres. Howe (74), using an independent centromere marker to detect misassortments, studied double exchanges across the centromere of the sex chromosome of *Neurospora crassa*. Although this was the same region for which the Lindegrens had earlier reported nonrandom crossing over, Howe's data when corrected for misassortments, fitted the 1:2:1 ratio expected on the basis of random crossing over. Stadler (75, 76), using Howe's method for detecting misassortments and an ascospore mutant which allowed scoring of asci by examination, reported that double exchanges across the centromere in linkage group VI of *Neurospora* were also in accordance with the random hypothesis. Lindegren & Shult (41) claim that Howe's data are too scanty to be significant and that Stadler, who accumulated much larger numbers, studied regions too long to reveal the locally specific areas showing chromatid interference reported by the Lindegrens. These authors, who feel that virtually all basic assumptions of classical genetics regarding crossing over are invalid, also claim that multiple crossovers occur in very short chromosomal regions and have published a series of papers on crossing over and tetrad analysis based on these ideas (41, 77, 78, 79). Weinstein (80) disagrees sharply with the views expressed by Lindegren and Shult on both theoretical and experimental grounds. He has pointed out that if multiple exchanges occur within short regions, then four-strand doubles should give rise to nonparental ditype tetrads in crosses involving closely linked markers. Investigators other than Lindegren and his co-workers, have not

obtained such results in extensive tetrad analyses carried out with a number of different fungi (37, 73, 81, 82).

Mathieson (83) has reinvestigated ascospore arrangements in *Bombardia lunata* in view of Zickler's previous findings which suggested polarized segregation of a gene affecting spore color. The same bias in first-division segregation classes was found but in second-division classes the bias was exactly reversed. Olive (37), working with *Sordaria fimicola,* and Bistis (84) with *Ascobolus stercorarius* reported that second-division segregation frequencies did not exceed the limit of 66.7 per cent imposed by the hypothesis of random crossing over. Data from tetrad analysis, used to construct linkage groups in *Glomerella* (82) and *Venturia* (81), also agreed with expectations based on the random hypothesis. Hawthorne (85) has utilized linear yeast asci to estimate gene-centromere distances and Leupold (86) has worked out segregation patterns for autotetraploids permitting tetrad analysis on the assumption that chiasma formation may occur at random between any two of four homologous chromosomes at any level. Whitehouse (87) has considered the mapping of centromeres in unordered tetrads including certain cases in which some of the loci are linked, and the use of tetratype frequencies for estimating spindle overlapping in ordered tetrads (88). Unpredictable and sometimes drastic effects of temperature changes on the frequency of recombinational events have been reported in *Sordaria* by Olive (37), in *Ascobolus* by Bistis (84), and in *Neurospora* by Stadler (75). Lindegren & Lindegren (89), using tetrad analysis, have studied eight genes controlling carbohydrate fermentation in yeast. In general, the conclusion reached by Perkins (73) in 1955, that nearly all data obtained by tetrad analysis can be satisfactorily accounted for on the hypothesis of random crossing over with positive chromosomal interference, still appears valid. Rare exceptions, even if supported by rigorous proof which has been lacking in most cases reported, do not provide justification for discarding the random hypothesis.

## Heterokaryosis

The genetic control of heterokaryosis in *Neurospora* has been studied by Holloway (90) and by Garnjobst (91). Results obtained by the former indicated that four, possibly five, genes controlled heterokaryon formation of a mutant requiring pantothenic acid and one requiring lysine. Several characters were affected including the time at which heterokaryotic growth started, the ability to maintain growth and the vigor of growth. Garnjobst (91) identified two gene pairs, *C c* and *D d* which control heterokaryon formation. She found that all nutritionally complementary pairs of cultures of the same mating type and like genotype at *C* and *D* formed heterokaryons (were $het^+$); whereas all other pairs failed to do so (were $het^-$) except certain *Cd* + *cd* pairs which occasionally gave slow growth. The action of the *C* and *D* genes was unrelated to the type of biochemical deficiency involved. In a further study, Garnjobst (92) reported that in $het^-$ combina-

tions protoplasmic incompatibility was evident with cells in the vicinity of hyphal fusions undergoing disintegration. Dowding & Bakerspigel studied nuclear migration in *Gelasinospora tetrasperma* and reported rates of migration as high as 10.5 mm. per hr. (93). They believe that the nuclei are not carried by the streaming of cytoplasm and suggest that, instead, they may be independently motile.

Atwood and his co-workers (94 to 97), have made an extensive study of nuclear mixing and the distribution of nuclear types in *Neurospora* heterokaryons. Mixtures of conidia were centrifuged to form a pellet which was then allowed to grow and produce conidia. Results obtained by plating conidia from such pellets showed that mixing was thorough and that the frequency of a given nuclear type was not influenced by the manner of its introduction, whether as a homokaryon or as a heterokaryon. They found, as had previous investigators, that the distribution of marked nuclei in macroconidia formed by heterokaryons was nonrandom; the departure taking the form of a tendency for like nuclei to occur together. Calculations based upon these results gave an approximation by which frequencies of a given nuclear type can be estimated from plate counts. The fact that heterokaryons often grow at the wild-type rate despite disproportions of nuclear components, had led previous workers to suggest that selection of faster growing hyphae having favorable nuclear ratios took place. Experiments with heterokaryons in which proportions of the nuclear components varied widely gave no evidence that such selection occurs (97).

*Cytoplasmic inheritance*—The effects of nuclear genes on two cytoplasmically inherited mutants in *Neurospora* has been studied by Mitchell & Mitchell (98). The two cytoplasmic mutants, *poky* and *mi-3* are similar in that both grow slowly and have defective cytochrome systems. A nuclear gene, *f*, restores growth to near normal in *poky* but does not restore its cytochrome defects and has no effect at all on *mi-3*. Two nuclear gene mutants, similar to *poky* in phenotype, also failed to respond to *f* but one of these nuclear mutants was restored to normal, both in growth and cytochrome, by a nuclear gene suppressor. This suppressor had no effect on any of the other nuclear or cytoplasmic mutants. Pittenger (99) and Gowdridge (100) studied heterokaryons of *poky*, *mi-3*, and wild-type in all possible combinations of cytoplasms. These studies led to the conclusion that *poky* and *mi-3* cannot interact to give wild-type and that no infective agent is involved.

In yeast, studies of respiratory-deficient mutants indicate that defects in genes or in cytoplasmic particles, or both, may be involved (101, 102, 103). Yčas (104) has reported that the hereditary cytochrome deficiency leading to "petite" colonies, known to be induced by euflavin, can also be induced by heat but only if growing cultures are exposed. According to James (105), spontaneous, respiratory-deficient mutants of nongenic origin, arise in yeast as the result of a sequence of chance events rather than through a breakdown in the orderly assortment of cytoplasmic particles.

Papazian (106), working with sectoring variants of *Schizophyllum,* discussed the difficulties of interpreting the variation as resulting from either genic or cytoplasmic factors and concluded that the former appears less satisfactory. Sectoring variants of *Aspergillus glaucus* studied by Sharpe (107) also yielded results which suggested that extranuclear, self-replicating particles were involved. The demonstration by Miles & Raper (108) that sodium taurocholate or cholic acids disrupt the process of clamp formation in many Basidiomycetes provides a technique which should be useful for studies of nuclear-cytoplasmic interactions in these fungi.

*Genetics of imperfect fungi.*—The model of vegetative segregation proposed for *Aspergillus nidulans* has been employed by Ishitani *et al.* (109 to 112) with *A. oryzae* and *A. sojae,* and by Sermonti (113, 114) with *Penicillium chrysogenum.* In both cases, the results obtained have paralleled earlier findings with *A. nidulans* (3). Results of a 10-year study of spontaneous and induced variation which produced the "Wisconsin family" of strains of *P. chrysogenum* have been summarized by Backus & Stauffer (115). They point out that during this period, strains were obtained which yielded ten times more penicillin than the improved strain from which the family arose and 40 times more than that produced by the wild-type ancestor. Caglioti & Sermonti (116) isolated several mutant strains of *P. chrysogenum* which failed to produce penicillin. Heterokaryons and diploids from combinations of such mutants did not produce penicillin but those involving one producing and one nonproducing strain gave yields equal to that of the producing strain.

Ishitani (117) has reported that ultraviolet radiation produced high frequencies of heterozygous diploids and somatic recombinants in two species of *Aspergillus.* These results were quite similar to those obtained earlier by James working with yeast (118). Studies of imperfect phytopathogens will be discussed in another section.

## Mutation

Space limitations permit inclusion of only a few of the many studies of spontaneous and induced mutation carried out with fungi. Kaplan (119) has discussed mutation and selection as methods for studying microorganisms, and Giles (120) has reviewed the extensive work on forward and reverse mutation in *Neurospora.* Kølmark & Giles (121) tested six monoepoxides for ability to induce reversions in a purple, adenineless mutant of *Neurospora.* They found that epoxyrings with side chains having strong electronegative properties were stronger mutagens than those with side chains having weaker electronegative charges. As a working hypothesis they proposed that negatively-charged centers are involved at the site of the mutations which react more readily with those epoxides carrying an increased positive charge on the terminal carbon atom of the epoxyring. Also with *Neurospora,* Woodward (122) reported that spontaneous mutation rates at several loci varied from 0.8 to 0.005 per $10^4$ conidia. Some very

interesting and rather spectacular morphological mutants in *Neurospora* have been described by Srb (123). Treatment of wild-type cultures with *beta*-propiolactone gave "melon" mutants which, upon transfer, sometimes yielded forms resembling mushrooms as the result of a second mutation. Similar mushroom-like forms were also obtained by forming heterokaryons of "melon" and aconidial strains.

An unstable strain of *Ustilago maydis* which mutated to require homocysteine at a high frequency ($1.4 \times 10^{-3}$ per sporidium per generation) has been studied by Ishikawa (124). Since revertants of such mutants were stable and low values for temperature coefficient and activation energy were found, the high rate of mutation was attributed to an unstable genic state rather than to the effects of a mutator. Holliday (125), working with the same fungus, has described an efficient method for the identification of biochemical mutants.

Curtis *et al.* (126) obtained acenaphthene-requiring strains of *Penicillium chrysogenum* by growing the fungus in the presence of this compound. Using similar methods, strains of yeast resistant to lithium chloride were obtained by Laskowski (127) who identified five genes, all dominant and at least three nonallelic, governing resistance. Srb (128), also working with yeast, found that red, adenine-requiring colonies give rise to white prototrophs if grown on media having a pH of 4.5 but not on media with a pH of 5.0. Since the reverse mutation was not affected by pH changes, the effect was attributed to rigorous selection of adenine-independent forms at the lower pH level.

Thomas *et al.* (129) studied the effects of several carcinogenic chemicals on mushrooms and reported that certain volatile mineral oils and coal-tar derivatives produced tumorous conditions and marked cytological changes in several of the fungi tested. An interesting variant of *Gelasinospora calospora,* which apparently arose as the result of mutations at two loci on the same chromosome, has been reported by Wilson & Alexopoulos (130).

## Genetics of Sexuality

Several important developments in this area have been reported since the subject was reviewed by Raper (131). Genetic and cytological reinvestigations of *Hypomyces solani* f. *curcurbitae* by El-Ani (132, 133) have provided clear evidence that sexual differentiation in this fungus is controlled by gene differences rather than by differences in chromosome numbers among various strains as had been claimed in earlier reports. Working with the same fungus, Baker (134) spermatized "female" cultures with mixtures of conidia from "males" and "hermaphrodites." The number of perithecia fertilized by spores from "males" varied with the age of the recipient culture and light conditions but was generally higher than the number fertilized by spores from "hermaphrodites." This was believed due at least in part to differences in the rates at which perithecia matured.

Sexual hormones, previously clearly demonstrated only in lower fungi,

have been reported in studies of two species of Ascomycetes. In *Glomerella cingulata,* two substances have been found in culture filtrates which repair genetically-blocked steps in the sexual process. [Driver & Wheeler (135); Wheeler (136)]. In *A. stercorarius,* Bistis (137, 138) has obtained evidence of hormonal mechanisms involved in the activation of fertilizing elements (oidia), the induction of ascogonia, and the directional growth responses of trichogynes.

Confusion and disagreement regarding the use and meaning of the terms "homothallism" and "heterothallism" prevail in spite of numerous attempts to arrive at satisfactory definitions. Burnett (139), in a review of mating systems in fungi, proposes to discard the terms entirely in favor of a new set which he has coined. Although this proposal has considerable merit, the fact that these terms have become firmly imbedded in the literature of several different disciplines makes it probable that they will continue to be used and misused. Much of the confusion regarding these terms can be attributed to the fact that a fungus thallus, even if initiated from a single, uninucleate, haploid spore, is not a genetically homogeneous entity. Apparent homothallic behavior may be exhibited by forms which are fundamentally heterothallic either as the result of mutations involving mating type or through the inclusion of two nuclei of opposite mating type in the same thallus. Dodge (140) has pointed out that for fungi such as *Neurospora tetrasperma,* which exhibit the latter type of behavior, the term "pseudohomothallic" is more appropriate than those previously employed. For this review, heterothallism is defined as a condition which requires that two genetically differentiated nuclei be brought together for completion of the sexual process, and homothallism as a condition in which such genetic differentiation is not a prerequisite for completion of the sexual process. Although these definitions provide a clear basis for separating two fundamentally different types of behavior, they are subject to the obvious objection that they do not coincide with the literal meanings of the terms. Perhaps two new terms, "homo-" and "heterokaryogamy," would be more satisfactory.

Despite the fact that the first case of hybridization among the higher fungi was reported by Edgerton in 1914 working with homothallic cultures of *Glomerella,* the notion has persisted that genetic studies with homothallic fungi cannot be carried out unless special procedures such as those used with *Aspergillus nidulans* are employed. Olive's work with *Sordaria* (137), together with extensive genetic analyses carried out with homothallic cultures of *Glomerella* (82), should serve to dispel this misconception. Induced selfing, the phenomenon in which nearly or completely self-sterile cultures become self-fertile in matings, previously known only in *Glomerella* (135, 141), has been reported in *S. fimicola* by Olive (37) and in *S. macrospora* by Esser & Straub (142). Olive attributes induced selfing in his material to diffusible chemical substances, whereas, Esser and Straub explain the phenomenon as the result of fusion of like nuclei in perithecia produced by heterokaryotic mycelia. Although the evidence available from studies of

*Glomerella* favors the former interpretation, it is entirely possible that both mechanisms may be involved.

In *Ustilago maydis* Rowell (143) has studied two loci: one with two alleles which governs fusion of compatible sporidia and one with multiple alleles which controls the vigor and stability of the dikaryon. Cantino (144) has reviewed the extensive studies of cellular morphogenesis and metabolism which have been carried out with *Blastocladiella*.

## Genetics of Phytopathogens

A general review of this subject is included in a recent book by Stakman & Harrar (145). In another book, Fischer & Holton (146) have reviewed the extensive genetic investigations carried out with the smut fungi. Kendrick's (147) discovery of certain haploid lines of *Tilletia caries* which complete their life cycle, including the production of teliospores, in artificial culture may provide an important new tool for genetic studies of this smut which causes a destructive disease of wheat. Keitt & Boone (148, 149) have reviewed the long series of papers which they and their coworkers have published on variability, inheritance, and pathogenicity of the apple scab fungus, *Venturia inaequalis*.

Flor's genetic analysis of host-parasite interactions with flax rust probably represents the most detailed study of this type ever carried out with a plant pathogen (150, 151). He interprets his data as indicating that for each gene controlling virulence or avirulence in the parasite there is a corresponding gene conditioning susceptibility or resistance in the host. Since designation of a parasite as virulent automatically infers that the host involved is susceptible, Flor's concept contains a redundancy which, at times, causes considerable confusion. LeRoux & Dickson (152) have initiated a genetic study of corn rust along lines similar to those followed by Flor. They have reported evidence of complex interactions and feel that the gene-for-gene hypothesis of the relationship between rust reaction in the host and pathogenicity in the parasite is an oversimplification.

The most interesting new development in regard to genetics of plant pathogens during the period under review is found in several reports of recombination of characters in the dikaryotic stage of rust fungi. Nelson *et al.* (153, 154) dusted mixtures of urediospores of two biotypes of *Puccinia graminis* var. *tritici* on susceptible wheat plants. The urediospores produced were transferred to resistant varieties of wheat and a form was obtained which was highly pathogenic to Khapli, a variety hitherto resistant to all known North American races of stem rust. This new form was unstable and, when single urediospores were isolated from it, both of the original parental biotypes and two new races were obtained. Cytological studies revealed that some of the urediospores of the unstable form contained three or four nuclei, whereas those from the stable biotypes had the usual dikaryotic constitution. This indicated that the unstable form represented a heterokaryon formed by the two original dikaryotic cultures and

that the new races had arisen as the result of dikaryon formation involving new combinations of nuclei. Further studies of new races arising from mixtures of uredial clones have been carried out with *P. graminus* var. *tritici* by Watson (155, 156) and with *P. recondita* var. *tritici* by Vakili & Caldwell (157). The number of new races obtained by the latter exceeded the number expected if only recombinations of intact nuclei were responsible. Further evidence, however, is required to determine whether some other mechanism such as gene conversion or somatic recombination is involved. Buxton's (158, 159) work with *Fusarium oxysporum* f. *pisi*, carried out by the methods developed for analysis of vegetative segregation with *Aspergillus*, suggest that these techniques may prove very valuable for genetic studies of imperfect fungi which cause plant diseases.

## LITERATURE CITED


1. Zelle, M. R., *Ann. Rev. Microbiol.*, **9**, 45–96 (1955)
2. Emerson, S., *Handbuch der physiol.- und pathologisch.-chemischen Analyse*, 10 Aufl., **Bd. II**, 443 (1955)
3. Pontecorvo, G., *Ann. Rev. Microbiol.*, **10**, 393–400 (1956)
4. Wagner, R. P., and Mitchell, H. K., *Genetics and Metabolism* (John Wiley & Sons, Inc., New York, N.Y., 444 pp., 1955)
5. Sussman, A. S., *Mycologia*, **49**, 29–43 (1957)
6. Teas, H. J., *Ann. Rev. Plant Physiol.*, **8**, 393–412 (1957)
7. Tatum, E. L., and Gross, S. R., *Ann. Rev. Physiol.*, **18**, 53–68 (1956)
8. Butler, J. A. V., and Davison, P. F., *Advances in Enzymal.*, **18**, 161–90 (1957)
9. DeBusk, A. G., *Advances in Enzymol.*, **17**, 393–476 (1956)
10. Beadle, G. W., in *The Chemical Basis of Heredity*, 3–22 (The Johns Hopkins Press, Baltimore, Md., 848 pp., 1957)
11. Glass, B., in *The Chemical Basis of Heredity*, 757–834 (The Johns Hopkins Press, Baltimore, Md., 848 pp., 1957)
12. Wilkins, M. H. F., *Cold Spring Harbor Symposia Quant. Biol.*, **21**, 75–90 (1956)
13. Delbruck, M., and Stent, G. S., in *The Chemical Basis of Heredity*, 699–736 (The Johns Hopkins Press, Baltimore, Md., 848 pp., 1957)
14. Lindegren, C. C., *Ann. N. Y. Acad. Sci.*, **69**, 338–51 (1957)
15. Winge, Ø., *Heredity*, **9**, 373–84 (1955)
16. Roman, H., Phillips, M. M., and Sands, S. M., *Genetics*, **40**, 546–61 (1955)
17. Leupold, U., *J. Genetics*, **54**, 411–26 (1956)
18. Roman, H., *Cold Spring Harbor Symposia Quant. Biol.*, **21**, 175–85 (1956)
19. Saito, H., and Takahashi, T., *Idengaku Zasshi* (Japan. J. Genetics), **32**, 147–52 (1957)
20. Lindegren, C. C., *Science*, **121**, 605–7 (1955)
21. Lindegren, C. C., Lindegren, G., Drysdale, R. B., Hughes, J. P., and Brenes-Pomales, A., *Genetica*, **28**, 1–24 (1956)
22. Ogur, M., and St. John, R., *Genetica*, **28**, 25–26 (1956)
23. Pittman, D. D., and Pedigo, P. R., *Genetica*, **28**, 27–30 (1956)
24. Emerson, S., *Compt. rend. trav. lab. Carlsberg Sér. physiol.*, **26**, 71 (1956)
25. Lindegren, C. C., and Lindegren, G., *Nature*, **178**, 796–97 (1956)

26. Winge, Ø., and Roberts, C., *Genetica*, **28**, 489–96 (1957)
27. Mitchell, M. B., *Proc. Natl. Acad. Sci. U. S.*, **41**, 215–20 (1955)
28. Mitchell, M. B., and Mitchell, H. K., *Genetics*, **41**, 305–18 (1956)
29. Mitchell, H. K., *The Chemical Basis of Heredity*, 94–113 (The Johns Hopkins Press, Baltimore, Md., 848 pp., 1957)
30. St. Lawrence, P., *Proc. Natl. Acad. Sci. U. S.*, **42**, 189–94 (1956)
31. De Serres, F. J. de, *Genetics*, **41**, 668–76 (1956)
32. Giles, N., de Serres, F., and Barbour, E., *Genetics*, **42**, 608–17 (1957)
33. Case, M. E., and Giles, N. H., *Genetics*, **42**, 364 (1957)
34. Stern, C., *Cold Spring Harbor Symposia Quant. Biol.*, **21**, 184 (1956)
35. Bonner, D. M., *Cold Spring Harbar Symposia Quant. Biol.*, **21**, 163–70 (1956)
36. St. Lawrence, P., and Bonner, D. M., *The Chemical Basis of Heredity*, 114–122 (The John Hopkins Press, Baltimore, Md., 848 pp., 1957)
37. Olive, L. S., *Am. J. Botany*, **43**, 97–107 (1956)
38. Pritchard, R. H., *Heredity*, **9**, 343–72 (1955)
39. Pontecorvo, G., *Cold Spring Harbor Symposia Quant. Biol.*, **21**, 171–174 (1956)
40. Sansome, E., *Compt. rend. trav. lab. Carlsberg Sér. physiol.*, **26**, 315–33 (1956)
41. Lindegren, C. C., and Shult, E. E., *Experientia*, **13**, 48–51 (1957)
42. Spiegelman, S., *The Chemical Basis of Heredity*, 131 (The Johns Hopkins Press, Baltimore, Md., 848 pp., 1957)
43. Benzer, S., *The Chemical Basis of Heredity*, 70–93 (The Johns Hopkins Press, Baltimore, Md., 848 pp., 1957)
44. Yanofsky, C., and Bonner, D. M., *Genetics*, **40**, 761–69 (1955)
45. Suskind, S. R., Yanofsky, C., and Bonner, D. M., *Proc. Natl. Acad. Sci. U. S.*, **41**, 577–82 (1955)
46. Yanofsky, C., *Methods in Enzymol.*, **2**, 233–38 (1955)
47. Suskind, S. R.., *J. Bacteriol.*, **74**, 308–18 (1957)
48. Suskind, S. R., and Kurek, L. I., *Science*, **126**, 1068–69 (1957)
49. Yanofsky, S., *Enzymes: Units of Biological Structure and Function*, 147–160 (Academic Press, Inc., New York, N.Y., 1956)
50. Suskind, S. R., *The Chemical Basis of Heredity*, 123–129 (The Johns Hopkins Press, Baltimore, Md., 848 pp., 1957)
51. Allen, J. P., and Lee-Whiting, B., *Genetics*, **40**, 826–31 (1955)
52. Pateman, J. A., and Fincham, J. R. S., *Heredity*, **10**, 279–80 (1956)
53. Parks, L. W., and Douglas, H. C., *Genetics*, **42**, 283–88 (1957)
54. Woodward, V. W., Munkres, K., and Suyama, Y., *Genetics*, **42**, 403 (1957)
55. Calef, E., *Heredity*, **10**, 279 (1956)
56. Calef, E., *Heredity*, **11**, 265–79 (1957)
57. Roper, J. A., and Kafer, E., *J. Gen. Microbiol.*, **16**, 660–67 (1957)
58. Pontecorvo, G., and Kafer, E., *Proc. Roy. Phys. Soc. Edinburgh*, **26**, 16–20 (1956)
59. Pontecorvo, G., and Roper, J. A., *Heredity*, **10**, 124 (1956)
60. Roper, J. A., and Pritchard, R. H., *Nature*, **175**, 639 (1955)
61. Robinow, C. F., *Can. J. Microbiol.*, **3**, 771–89 (1957)
62. Robinow, C. F., *Can. J. Microbiol.*, **3**, 790–98 (1957)
63. Girbardt, N., *Naturwissenschaften*, **43**, 429–30 (1956)
64. Haldane, J. B. S., *The Biochemistry of Genetics* (George Allen & Unwin, Ltd., London, Eng., 144 pp., 1954)
65. Horowitz, N. H., *Federation Proc.*, **15**, 818–22 (1956)

66. Horowitz, N. H., and Fling, M., in *Enzymes: Units of Biological Structure and Function,* 139–45 (Academic Press., Inc., New York, N.Y., 1956)
67. Lederberg, J., in *Enzymes: Units of Biological Structure and Function,* 161–69 (Academic Press, Inc., New York, N.Y., 1956)
68. Mathieson, M. J., and Catcheside, D. G., *J. Gen. Microbiol.,* **13,** 72–83 (1955)
69. Winge, Ø., and Roberts, C., *Compt. rend. trav. lab. Carlsberg Sér. physiol.,* **25,** 420–57 (1957)
70. Losada, M., *Compt. rend. trav. lab. Carlsberg Sér. physiol.,* **25,** 460–81 (1957)
71. Fincham, J. R. S., and Pateman, J, A., *Nature,* **179,** 741 (1957)
72. Spiegelman, S., in *The Chemical Basis of Heredity,* 232–67 (The Johns Hopkins Press, Baltimore, Md., 848 pp., 1957)
73. Perkins, D. D., *J. Cellular Comp. Physiol.,* Suppl. 2., **45,** 119–49 (1955)
74. Howe, H. B., Jr., *Genetics,* **41,** 610–22 (1956)
75. Stadler, D. R., *Genetics,* **41,** 528–43 (1956)
76. Stadler, D. R., *Genetics,* **41,** 623–30 (1956)
77. Shult, E. E., and Lindegren, C. C., *Genetica,* **28,** 165–76 (1956)
78. Shult, E. E., and Lindegren., C. C., *J. Genet.,* **54,** 343–57 (1956)
79. Shult, E. E., and Lindegren, C. C., *Nature,* **179,** 683 (1957)
80. Weinstein, A., *Genetics,* **42,** 401 (1957)
81. Boone, D. M., and Keitt, G. W., *Am. J. Botany,* **43,** 226–33 (1956)
82. Wheeler, H. E., *Am. J. Botany,* **43,** 1–6 (1956)
83. Mathieson, M. J., *Ann. Botany* (*London*), **20,** 623–34 (1956)
84. Bistis, G., *Bull. Torrey Botan. Club,* **83,** 35-61 (1956)
85. Hawthorne, D. C., *Genetics,* **40,** 511–18 (1955)
86. Leupold, U., *J. Genet.,* **54,** 427–39 (1956)
87. Whitehouse, H. L. K., *J. Genet.,* **55,** 348–60 (1957)
88. Whitehouse, H. L. K., *Nature,* **179,** 162 (1957)
89. Lindegren, C. C., and Lindegren, G., *J. Gen. Microbiol.,* **15,** 19–28 (1956)
90. Holloway, B. W., *Genetics,* **40,** 117–29 (1955)
91. Garnjobst, L., *Am. J. Botany,* **42,** 444–48 (1955)
92. Garnjobst, L., *Proc. Natl. Acad. Sci. U.S.,* **42,** 613–18 (1956)
93. Dowding, E. S., and Bakerspigel, A., *Can. J. Microbiol.,* **1,** 68–78 (1955)
94. Atwood, K. C., and Mukai, F., *Genetics,* **40,** 438–43 (1955)
95. Atwood, K. C., and Pittenger, T. H., *Am. J. Botany,* **42,** 496–500 (1955)
96. Pittenger, T. H., Kimball, A. W., and Atwood, K. C., *Am. J. Botany,* **42,** 954–58 (1955)
97. Pittenger, T. H., and Atwood, K. C., *Genetics,* **41,** 227–41 (1956)
98. Mitchell, M. B., and Mitchell, H. K., *J. Gen. Microbiol.,* **14,** 84-89 (1956)
99. Pittenger, T. H., *Proc. Natl. Acad. Sci. U.S.,* **42,** 747–51 (1956)
100. Gowdridge, B. M., *Genetics,* **41,** 780–89 (1956)
101. Pittman, D. D., *Exptl. Cell Research,* **11,** 654–55 (1956)
102. Ogur, M., and St. John, R., *J. Bacteriol.,* **72,** 500–4 (1956)
103. Lindegren, C. C., Ogur, M., Pittman, D. D., and Lindegren, G., *Science,* **126,** 398–99 (1957)
104. Yčas, M., *Exptl. Cell Research,* **10,** 746 (1956)
105. James, A. P., *Genetics,* **42,** 379 (1957)
106. Papazian, H. P., *Am. J. Botany,* **42,** 394–400 (1955)
107. Sharpe, H. S., *Heredity,* **10,** 280 (1956)

108. Miles, P. G., and Raper, J. R., *Mycologia,* **48,** 484–95 (1956)
109. Ishitani, C., and Sakaguchi, K., *J. Gen. Appl. Microbiol.,* **1,** 246–57 (1955)
110. Ishitani, C., and Sakaguchi, K., *J. Gen. Appl. Microbiol.,* **1,** 258–71 (1955)
111. Ishitani, C., and Sakaguchi, K., *J. Gen. Appl. Microbiol.,* **1,** 283–97 (1955)
112. Ishitani, C., and Sakaguchi, K., *J. Gen. Appl. Microbiol.,* **2,** 345–400 (1956)
113. Sermonti, G., *J. Gen. Microbiol.,* **15,** 599–608 (1956)
114. Sermonti, G., *Genetics,* **42,** 433–43 (1957)
115. Backus, M. P., and Stauffer, J. F., *Mycologia,* **47,** 429–63 (1955)
116. Caglioti, M. T., and Sermonti, G., *J. Gen. Microbiol.,* **14,** 38–46 (1956)
117. Ishitani, C., *Nature,* **178,** 706 (1956)
118. James, A. P., *Genetics,* **40,** 204–13 (1955)
119. Kaplan, R. W., *Arzneimittel-Forsch.,* **7,** 280 (1957)
120. Giles, N. H., *Brookhaven Symposia in Biol., No. 8(BNL350(C-22)),* 103–25 (1956)
121. Kølmark, G., and Giles, N. H., *Genetics,* **40,** 890–902 (1955)
122. Woodward, V. W., *Proc. Natl. Acad. Sci. U.S.,* **42,** 752–57 (1956)
123. Srb, A. M., *J. Heredity,* **48,** 146–53 (1957)
124. Ishikawa, T., *Idengaku Zasshi* (Japan. J. Genetics), **32,** 213–22 (1957)
125. Holliday, R., *Nature,* **178,** 987 (1956)
126. Curtis, R. W., Stauffer, J. F., and Backus, M. P., *Am. J. Botany,* **43,** 594–600 (1956)
127. Laskowski, W., *Genetics,* **41,** 98–106 (1956)
128. Srb, A. M., *Genetics,* **42,** 398 (1957)
129. Thomas, P. T., Evans, H. J., and Hughes, D. T., *Nature,* **178,** 949–51 (1956)
130. Wilson, G. B., and Alexopoulos, C. A., *Mycologia,* **48,** 685–89 (1956)
131. Raper, J. R., *Sex in Microorganisms,* 42–81 (Am. Assoc. Advance. Sci., Washington, D.C., 362 pp., 1954)
132. El-Ani, A. S., *Am. J. Botany,* **41,** 110–13 (1954)
133. El-Ani, A. S., *Am. J. Botany,* **43,** 769–78 (1956)
134. Baker, R., *Phytopathology,* **45,** 644–49 (1956)
135. Driver, C. H., and Wheeler, H. E., *Mycologia,* **47,** 311–16 (1955)
136. Wheeler, H. E., *Mycologia,* **48,** 349–53 (1956)
137. Bistis, G., *Am. J. Botany,* **43,** 389–94 (1956)
138. Bistis, G., *Am. J. Botany,* **44,** 436–43 (1957)
139. Burnett, J. H., *New Phytologist,* **55,** 50–90 (1956)
140. Dodge, B. O., *Bull. Torrey Botan. Club,* **84,** 182–88 (1957)
141. Wheeler, H. E., *Assoc. Southeast. Biol. Bull.,* **1,** 12 (1955)
142. Esser, K., and Straub, J., *Z. Induktive Abstammungs-u. Vererbungslehre,* **87,** 625–26 (1956)
143. Rowell, J. B., *Phytopathology,* **45,** 370–74 (1955)
144. Cantino, E. C., *Mycologia,* **48,** 225–40 (1956)
145. Stakman, E. C., and Harrar, J. G., *Principles of Plant Pathology,* 121–77 (The Ronald Press Co., New York, N.Y., 581 pp., 1957)
146. Fischer, G. W., and Holton, C. S., *Biology and Control of the Smut Fungi* (The Ronald Press Co., New York, N.Y., 622 pp., 1957)
147. Kendrick, E. L., *Phytopathology,* **47,** 674–76 (1957)
148. Keitt, G. W., and Boone, D. M., 125–29 in *Therapy of Fungus Diseases* (Sternberg, T. H., and Newcomer, V. D., Eds., Little, Brown & Co., Boston, Mass., 1955)

149. Keitt, G. W. and Boone, D. M., *Brookhaven Symposia in Biol., No. 9(BNL350(C-22))*, 209–23 (1956)
150. Flor, H. H., *Phytopathology*, **45**, 680–85 (1955)
151. Flor, H. H., *Advances in Genet.*, **8**, 29–54 (1956)
152. LeRoux, P. M., and Dickson, J. G., *Phytopathology*, **47**, 101–7 (1957)
153. Nelson, R. R., Wilcoxson, R. D., and Christensen, J. J., *Phytopathology*, **45**, 639–43 (1955)
154. Nelson, R. R., *Phytopathology*, **46**, 538–40 (1956)
155. Watson, I. A., *Phytopathology*, **47**, 507–9 (1957)
156. Watson, I. A., *Phytopathology*, **47**, 510–12 (1957)
157. Vakili, N. G., and Caldwell, R. M., *Phytopathology*, **47**, 536 (1957)
158. Buxton, E. W., *Biochem. J.*, **63**, 30–31 (1956)
159. Buxton, E. W., *J. Gen. Microbiol.*, **15**, 133–39 (1956)

# REVIEW OF THE MICROBIOLOGICAL AND IMMUNOLOGICAL LITERATURE PUBLISHED IN 1957 IN THE U.S.S.R.[1]

By Pierre Grabar[2]
*Institut Pasteur, Paris, France*

The same aims, presentation, and limitations found in the previous reviews on this subject are maintained in the present article. The whole problem of scientific literature in the Russian language has recently been envisaged by Adams (1), and it seems to the present author that the manner in which he is presenting these reviews (i.e., information which is as complete as possible without discussion) is justified and fits well into the general objective. The availability of complete translations of a series of Russian scientific journals will be useful. Among these journals are the *Journal of Microbiology, Epidemiology, and Immunobiology* which is completely devoted to the subject under review, and *Biokhimiya* which often contains articles on microbial chemistry, immunochemistry, etc. Thus, the reader of this review may find the complete translations of those articles in which he has a particular interest and these are only mentioned here.

In last year's review, the appearance of two new journals was announced: *Voprosy Virusologii* and *Antibiotiki*. Some of the articles published in these journals in 1957 are cited in this review. The first of these journals (bimonthly) is concerned with virology and contains original articles, reviews on general problems, and discussions on practical questions. *Antibiotiki* (also bimonthly) is concerned with the study of antibiotics and contains descriptions of new antibiotic substances, their activities, and methods for their preparation, but most of the articles are devoted to practical applications.

In 1957, the total number of publications on the subjects of this review has increased, but the number of those which are cited here remains unchanged. Thus, the author was obliged to exclude more articles than previously (about two-thirds).

The general problems or trends in research do not seem to have changed greatly, and practical applications still predominate. Fewer publications on the role of the nervous system in infectious diseases, and in immunity appeared in 1957, but the discussion of this question is continued. Among the infectious diseases, dysentery is certainly the most studied and a large

[1] The survey of the literature pertaining to this review was concluded in January, 1958. Some of the last 1957 issues of the reviewed journals had not been received in Paris at that time.

[2] The author expresses his thanks to Miss Dorothy Lane for her help in correcting the English text of this review and to Mrs. Nina Grabar and Miss Ludmila Grabar for their contribution in assembling the bibliography.

number of investigations on its epidemiology and control, as well as on the isolation of variant strains and on the attempts to produce active prophylactics, have been performed.

As previously, each issue of the *Journal of Microbiology, Epidemiology and Immunobiology* is concerned mainly with one general problem, although it also contains articles on other questions. Many publications are only short notes, called "autoreferat," i.e., a summary written by the author, which generally contains no descriptions of the methods used and no discussion of results. The delay in publications in most of the journals seems to be about 6 to 12 months.

## Systematics, Morphology, Variations

A thermophilic bacterium capable of fixing molecular nitrogen has been isolated from a congealed soil; it is aerobic and exhibits optimum growth at 45 to 50°C.; and its nitrogen fixing properties resemble those of *Clostridium pasteurianum* (2). Chernomordik and his associates (3) propose to include *Bacillus pyoceaneus* and *Malleomyces mallei* in the genus *Pseudomonas* because of serologic relationships.

Six strains of cytopathogenic adenoviruses have been isolated and cultivated on cell cultures *in vitro*. After 5 successive transfers on cultures of HeLa cells, they have preserved their characteristic cell-degenerating activity (4). A serologic study of the virus causing the disease called "dikovanie" (the "wildness") which exists in regions to the north of the polar circle, showed that it is a variant of rabies; the variation is supposed to have appeared as a result of natural conditions in this region (5).

The polyhedric viruses which form inclusions in cell nuclei of the caterpillar of *Galleria mellonella* have been studied by electronic and ordinary microscopy. The polyhedres are 2μ large and have a nearly regular cubic structure. When treated with a dilute base, they decompose into rod-shaped particles, the dimensions of which are about 300 × 30 mμ. Gershenzon (6) proposes giving this virus the provisional name of *Borrelina galleriae* n. sp, but he insists that it is difficult to decide whether the existence of different species may be admitted.

Protsenko (7) has given a description of several plant diseases, mainly mosaics caused by viruses, observed on 73 species belonging to 22 genera in the Main Botanic Garden. Fedotina concludes from her experiments that the crystalline inclusions observed in the potato, which have been identified as nucleoproteins, are not necessarily virus particles or produced during virus disease (8).

The question of bacterial viruses has been the subject of several publications, two of which were general reviews (one in *Voprosy Virusologii* and the other in *Usepkhi Sovremennoĭ Biologii*); the others were concerned with morphologic or toxinogenic modifications of the bacterial cell under the action of phages, the use of phages in typing bacterial strains, the stability of phage suspensions, etc. In order to obtain active, stable prepara-

tions of dysenteric phages, the choice of bacterial strains particularly rich in whole antigen is recommended, although no direct relationship could be established between the quantity of this antigen (measured by the polysaccharide content of the microbes) and the virulence of the strain (9). Fifty-three strains of *Brucella* phages have been isolated and their properties studied. Most of them were only active on a particular microbial strain, but some phages lysed even the three species of *Brucella*. Freshly isolated strains of phages generally possess a low activity, but their titers could be increased to $10^9$ after several passages. The activity was more pronounced when tested on freshly isolated strains of *Brucella* than on laboratory strains (10).

Studies on the flagella of 20 different strains of bacteria isolated from the nodules of alfalfa have been performed by electron microscopy, and it has been found that all of them have only one flagellum inserted at one of the poles of the microbe (11).

Various structures have been observed in cultures of influenza type D and bird plague viruses: large, spherical bodies and filamentous particles, some of them with enlargements. Draganov (12) assumes that certain structures represent intermediate states and that the large bodies grow out from elementary particles.

Synthesis of infective polyhedric viruses from their noninfective degradation products has been achieved. A solution of the sodium salt of the deoxyribonucleic acid extracted from the virus was mixed with a solution (pH 8) of the virus protein (obtained by extraction with sodium carbonate); the mixture was maintained for 4 hr. at 4°C. and then for 3 hr. at room temperature (13).

The initial stages of development of the tobacco mosaic virus have been investigated by Sukhov (14). During the latent period of infection, formation of fibrillar structures was observed. The presence of fibrillae of various lengths seems to suggest that they are growing by increase in length. The formation of spirals from these particles explains the increase of the diameter of the mature rods and the modification of their antigenic properties.

At least 40 publications can be grouped under the general heading of variation (including mutations, adaptations, and dissociations). As in the preceding years, many articles contain descriptions of atypical strains of bacteria, mainly of the enteric group and particularly of *Shigella*, some of which showed common antigens or common biochemical reactions with other bacteria, largely of the *Escherichia coli* group. The survival time or the persistence of different strains under various conditions in natural waters and in the presence of chlorine has been studied, and under these conditions some authors have observed the appearance of variants.

Vasilenko (15) claims to have been able, by successive cultures on a poor, solid medium and a selection of colonies, to obtain several different strains of *E. coli* which had lost some of their biochemical activities, had

become agglutinable by anti-Flexner sera, and could be lysed by dysenteric phages, and one strain which was agglutinable by an anti-paratyphi B serum. These strains maintain their properties, and Vasilenko admits that his technique allowed him to induce the formation of stable variants.

Modifications of microbial strains under the action of "transforming substances" have been reviewed by Loginova; the nature of the substances used (i.e., the DNA of other microbes), the conditions in which the transformation may be obtained, the properties of the new strains (virulence, resistance to chemical agents, capacity to ferment different sugars, etc.) have been described (16).

When *E. coli* was cultivated in a culture of leucocytes supplemented with whole antigen from *Salmonella schottmuelleri* (paratyphi B), variants were obtained which were similar to this latter microorganism in their biochemical, pathogenic, and antigenic properties (17). A modification of a *Shigella flexneri* strain during its culture on leucocytes has been observed after the eighth passage (one every week), while the same strain cultivated on other media maintained its properties (18).

Strains of *Shigella sonnei* and *S. flexneri* resistant to streptomycin, synthomycin, levomycetin, and biomycin have been obtained, but studies on their morphologic, serologic, biochemical, and pathogenic properties have not permitted Nobokov to observe any significant variations. Vaccines prepared from the resistant strains were as effective against the original, as against the resistant strains (19). However, Moroz claims that strains of *Shigella dysenteriae* (Shiga) and *S. flexneri* resistant to grisemin show a diminution or even a loss of specific agglutination, and an attenuation of their virulence and toxicity, as well as of their property to be lysed by specific phages (20).

Strains of *Proteus morganii,* adapted to synthomycin contain more DNA, and those adapted to streptomycin, less DNA than the original strain. The resistant strains were capable of utilizing some amino acids (glycine, alanine, leucine, lysine, and β-phenylalanine) which are not metabolized by the original strain, but were generally incapable of producing indole and gases on classical media; some of the adapted strains produced a carotenoid pigment (21).

Using the technique of single cell culture, Martyniuk has observed the formation of haemolytic variants from *Streptococcus viridans* and suggests that the appearance of the same kind of variation could also occur in the human organism (22).

The isolation of a strain of *Vibrio cholerae* virulent for guinea pigs from a nonvirulent strain has been achieved using chick embryo as culture medium. After a series of 20 to 30 passages, a strain was obtained which regularly killed guinea pigs of 220 to 250 gm. with a dose of 3 billions cells (23).

A strain of *Rickettsia prowazeki* has been maintained for 13 years on lice (440 passages) without visible modifications other than the loss of its cap-

sule. However, it became avirulent for guinea pigs, and in this animal even very large doses produced only a small increase in temperature lasting one day. All of the injected animals showed high agglutination or complement-fixation titers and were resistant to experimental infection attempted 40 days later; thus, this strain seems to be able to serve as a living vaccine (24).

When treated with ultrasonic waves under certain conditions, some microorganisms were transformed into variants possessing new properties. From a culture of the multicellular bacterium, *Caryophanon latum,* after such a treatment, a strain was isolated showing a different morphologic structure which persisted after five generations. When suspensions of *Hemophilus pertussis* were treated with ultrasonic waves and then maintained at 4°C. in the presence of their degradation products, this procedure being repeated five or six times, a strain possessing modified morphologic, toxic, and antigenic properties was obtained (25).

According to Filippovich, the virus of foot-and-mouth disease possesses a particular plasticity in its antigenic structure, and variations of its types can be observed, for example, in the guinea pigs with types A and C. In cattle, the modifications occur even faster, particularly with variants. If an immunized animal is infected several times with the same strain, various strains with different antigenic properties can be isolated (26).

The problem of variability of phytopathogenic viruses and, particularly, the hereditary persistence of new variants has been discussed by Sukhov (27).

The problem of filterable forms of microorganisms, the L- and G-forms, continues to be studied by several authors and was the subject of a series of publications, mainly in the *Zhurnal Microbiologii, Epidemiologii i Immunobiologii.* In most cases, these forms were obtained through the action of antibiotics, and the authors describe the successive stages of transformations with different strains of enteric bacteria (*E. coli,* typhoid and paratyphoid bacteria, and *Shigella*). In some cases, L-colonies have been obtained and different methods of regeneration have been described. Generally, the destruction of the original cells leaves granular particles but sometimes filamentous forms have also been observed. Culture of these granules on suitable media may regenerate the original microorganism. However, in some cases the formation of atypical strains has been observed, such as, for example, the appearance of atypical strains resembling *S. schottmuelleri* or *E. coli* from *S. typhosa* (28).

Filterable forms of diphtheria bacteria are said to have been obtained from the washings of infected tissues. When the filtrate of these fluids was injected intracerebrally into white mice, a small number of secondary cultures possessing typical properties of *Corynebacterium diphtheriae* and with an increased virulence could be isolated (29). On the other hand, a stable avirulent strain of *Mycobacterium tuberculosis* has been isolated from regenerated cultures of filterable forms of this microorganism, and this strain showed high immunogenicity (30). Levashev has studied the filter-

able forms of *Vibrio metchnikovii* and of *Proteus vulgaris* and compared the morphologic, biochemical, and serologic properties of the regenerated strains and the G- and L-forms. From these studies he concludes that the regenerated filterable forms and the G-forms are identical, that they seem to result from a kind of recombination of cell degradation products, and are frequently accompanied by a loss of some properties; whereas the L-forms are produced, not by destruction, but by an adaptation of the cells to special conditions (presence of penicillin) and generally revert to the original strain when cultivated in an appropriate medium. He observed also that in some cases the L-form of *Proteus,* when introduced into white mice, reverts to the original strain but with increased virulence (31, 32).

For many years, different authors have envisaged the existence of a special "filterable scarlatineous agent," sometimes designated by AC and KD. Konosh has compared the properties of these agents with those of the infectious ectromelia virus. Clinical symptoms and pathologic modifications in experimentally infected mice, as well as pathologic modifications in chick embryos, have been studied. Serologic reactions and cross-neutralization with specific immune sera and with sera from children (convalescent after scarlatina), previously immunized or nonimmunized with vaccinia virus, have been performed. The results obtained have shown that the so-called strains AC and KD of the "filterable scarlatineous agent" are identical with the virus of mice ectromelia in their morphologic, biologic, and antigenic properties. The capacity of human sera to neutralize these strains can be explained by the known cross reaction between vaccinia and ectromelia viruses (33).

## Biology of Microorganisms

*Constituents, metabolism, enzymatic activity.*—Three different protein fractions have been isolated from *C. diphtheriae;* their ultraviolet absorption spectra showed some differences, due certainly to quantitative differences in their cyclic amino acid content (34). Studies on the N-containing constituents of *Azotobacter* have been performed by Belozerskiĭ *et al.,* and the first published results show that *A. agile* and *A. vinelandii* are very similar in their contents, whereas *A. chroococcum* differs from them because it is rich in non-nitrogeneous substances. The relative quantities of ribonucleic acid (RNA) show large variations during the growth of the microbes and to a certain extent depend upon the culture medium. A correlation has been observed between the capacity of cells to multiply and the content of RNA (35). The same author with other associates has published a very detailed study of nucleic acids from 19 different microbial species. Using paper chromatography and ultraviolet absorption spectrophotometry, they showed that the ratio of the sum of guanylic and cytidylic acids to the sum of adenylic and thymylic acids in DNA varies for different species from 0.45 to 2.73 and that, as a general rule, related species possess a similar ratio. On the other hand, RNA extracted from the same microbes presents a much more

constant composition, although some small but significant differences seem to exist when unrelated species are compared. No definite correlation could be established between the relative composition of DNA and RNA in different species, but the authors think that such a correlation may exist, since a certain general tendency seems to be indicated by their results (36).

Several articles have been published on the isolation, purification, and properties of different microbial antigens, such as the whole antigen of *Shigella,* the Vi-antigen of *Salmonella typhosa,* the pertussis, streptococcal, and tetanus toxins, etc. Increase of yields or diminution of toxicity have been obtained in some cases.

The chemical composition of microbial cells and of isolated antigens from a variant of *E. coli,* obtained by cultivating this microorganism on heat-killed *S. typhosa* and *S. schottmuelleri* cells, has been studied. The analysis of the microbial cells showed that the content of N, P, and reducing sugars was different in the variant as compared to the initial strains. The amino acid composition of the protein fractions was qualitatively (chromatography) similar, but the analysis of the polysaccharide fractions showed that this fraction of the variant strain was analogous to that of the killed microbes used, and different from the original *E. coli* strain by the presence of ribose and of two unidentified sugars (37).

Two general reviews on the theory of respiration of microorganisms have been published by Fedorov. He insists particularly on the accumulation of energy, produced by oxidations, in the form of macroenergetic phosphate bonds. He admits that 44 bonds of this kind could be formed and, that if each of them corresponds to 12,000 calories, the total available energy must be equal to 528,000 calories per mole of hexose metabolized. This value would correspond to 65 to 75 per cent of the total energy of oxidation (38).

Seliber (39) proposes to introduce a slight modification in the general scheme for the $CO_2$ assimilation by heterotrophs, in admitting the formation of a hydrate of carbon dioxide.

A series of articles has been published in *Mikrobiologiya* in which the question of chemosynthesis was discussed. Most of the authors support the theory of Winogradsky and criticize some of Kalinenko's opinions. This author claims that he has observed an important and prolonged development of microbes in freshly prepared distilled water and supposes that the necessary metabolites penetrate into the medium with air through the cotton-plug (40). Growth and multiplication of *E. coli, Pseudomonas aeruginosa,* and *Proteus vulgaris* on Winogradsky's medium, even without ammonium sulfate and $CO_2$, has been observed and the same explanation (i.e., the penetration of volatile organic substances from the air into the culture medium) has been admitted (41). In experiments in which Winogradsky's medium was also used but in conditions which exclude possible penetration of organic substances from the atmosphere, it has been shown that *Nitrosomonas europaea* can use $CO_2$ as sole source of carbon (42). Experiments performed with radioactive sucrose or carbonate have shown that *Nitrosomonas* as-

similates the carbon from carbonates to build its organic constituents (43).

Studies on the intensity and on the wave lengths of light which are most favorable for the development of purple bacteria (*Rhodopseudomonas palustris*) have been performed by Maksimova (44); when higher intensities of light are used, the role of $CO_2$ as a source of carbon is increased, but with very high intensities heterotrophic assimilation is inhibited. Optimum growth is obtained with light of wavelengths longer than 790 mμ. The color of the microbes changes depending on the range of the spectrum utilized (44).

Metabolic studies on variant strains of *S. schottmuelleri* "regenerated" from a filtrate showed that it was incapable of fermenting sugars, but it could deaminate and oxidize some amino acids and also oxidize pyruvate, etc. In some cases, during the process of regeneration, a tendency to ferment sugars was noted (45).

Cultivation of different strains of the plague microbe on synthetic media permitted the demonstration that phenylalanine, methionine, cystine, serine, and threonine were required, whereas proline and tryptophan are only accessory factors. Using labelled cystine and methionine, it could be observed that the microbe used cystine for the formation of methionine, and that this transformation is irreversible (46).

Bacterial protoplasts from *Micrococcus lysodeikticus* have been prepared using lysozyme, and the rate of incorporation of labelled amino acids, as well as the protein nitrogen, respiration, and phosphate binding by these protoplasts, have been determined. The results obtained show that the rate of incorporation of labelled glycine depends on the structure of the protoplasts and is larger for less degraded structures. A parallel increase of protein nitrogen was also observed, but the authors admit that it may not necessarily be due to a real biosynthesis of proteins (47).

The role of Fe in the metabolism of *Clostridium acetobutylicum* has been investigated using labelled iron, $Fe^{59}$. It has been observed that in the presence of reduced iron or its salts, the yield of neutral products of the fermentation, and particularly of acetone and butyl alcohol, was increased. The metal is firmly bound by the microbe, but the quantity of it necessary for this anaerobic species is smaller than that which is used by *E. coli* or by purely aerobic bacteria (48).

Studies on the growth of bacteria from the nodules of alfalfa have shown that the sap of this plant is favorable for their development, but only if it is obtained from plants just before or during the period of flowering (49).

Substances stimulating or inhibiting the multiplication of tobacco mosaic virus, and the role of different cations on its multiplication, have been investigated. Thus, for example, sucrose, glucose, and certain organic acids stimulate the multiplication of the virus; whereas β-glycerophosphate and, beginning with a certain concentration, adenosine triphosphate (ATP) inhibit it. The amino acids, although favorable for the respiration of the tobacco leaves, diminish the multiplication of the virus. Phosphatase activity

seems to be important for the development of the virus. Acridine derivatives appear to be very potent inhibitors (50).

Lomteva has observed that, in some pathologic states, microbes of the normal human microflora may form variants capable of producing hyaluronidase (51).

Studies on the enzymatic properties of *Rickettsia prowazeki* have shown that this organism does not oxidize glucose, oxalic, succinic, or lactic acids, but does oxidize glutamic acid (52).

The culture medium of *Clostridium oedematiens* contains a very active and thermostable α-amylase (opt. pH = 6.9 to 7) which can be activated by resorcinol and inhibited by hydroquinone. The transformation of this medium into toxoid does not destroy the amylase activity (83 per cent of it persists). The cells of this microorganism contain, moreover, a fructosidophosphate aldolase, an α-glucosidase (opt. pH = 6.8 to 7.1), and a phosphorylase (opt. pH = 6.7 to 6.9), (53). The presence of α-amidases, and acylases of amino acids in *B. cadaveris* and a *Micrococcus* has been demonstrated. The deamination of *l*-tyrosineamide and of *l*-phenylalanineamide has been studied. This reaction is activated by Mg and Mn ions and inhibited by phosphate, Zn, Ca, Sn, Al, and Fe ions. The hydrolysis of *N*-formylglycine, of *N*-acetyl tyrosine and of hippuric acid has been observed, whereas *N*-acetylaspartic acid and *N*-chloracetylaspartic acid are not hydrolyzed by these microbes (54, 55). Twenty-eight different strains of dysenteric bacteria have been used in studies on histidine, tyrosine, arginine, lysine, and glutamic acid decarboxylases. The highest activity was observed with the last of these amino acids (56). The oxidation and deamination of purines and of nucleic acids by *S. schottmuelleri,* and by strains regenerated from filtrates of this microbe, have been investigated. The adenase appeared to be the most active of the envisaged enzymes. Among the regenerated strains, some had a higher and others a lower activity than that of the original strain (57).

Molybdenum seems to play an important role in the oxidation of nitrite by the nitrifying bacteria and is included in the flavoprotein complex (58).

*The action of bactericidal and antibiotic agents and substances.*—Investigations on the combined action of ultraviolet light ($\lambda = 2537$ Å) and x-rays on the spores of *Bacillus anthracoides* have shown that the produced effect was stronger than the calculated sum of the actions observed with each of these agents used separately (59). Somewhat analogous results have been obtained in studies on the combined *in vitro* effect of radioactive phosphorus and antibiotics on *E. coli, S. flexneri* and staphylococci. These microbes were cultivated on agar media containing streptomycin and $P^{32}$ in concentrations which, taken separately, had no visible effects; but when used simultaneously, gave rise to abnormal colonies and R-forms (60). Small amounts of β-radiation produced an increase in the yield of nodule bacteria; 1 to 2 mc./liter of solid medium and 5 mc./liter of liquid medium were used. Larger doses inhibited their growth (61).

The resistance of various strains of tick-induced encephalitis viruses to the action of the following chemical and physical agents has been described: heat, cold, ultraviolet light, alcohol, ether, phenol, trichloroacetic acid, formol. Some of the strains are still infectious after seven years' preservation in a dried state (62).

A new antibiotic substance, named actinoidin, has been isolated by Shorin *et al.* from culture media of *Proactinomyces actinoides*; it is a polypeptide and is active on Gram-positive bacteria and on *Spirochaeta hispanica*. This substance, although of low toxicity, produces painful reactions in the site of injection (63). Using a very large number of different strains of various *Shigella*, D'iakov has studied their antibiotic activity on other bacteria, and has observed that few strains (4 of 49 studied) of *S. flexneri* produced substances active on most of the strains of *E. coli*, *Salmonella typhosa*, *Shigella sonnei*, and *Salmonella suipestifer* which were studied. Strains belonging to the same species and serologic type may possess or be devoid of this activity (64). Several articles have been published on *Actinomycetes*: isolation of new strains or variants, activities on different microbes or fungi, methods of culture, morphologic aspects, etc. A series of different strains has been utilized for the extraction of grisin (or grisemin), which seems to be a polypeptide containing 14 to 14.5 per cent N (three-quarters of it being basic). This substance has a large spectrum of activity on many bacteria, yeasts, and fungi, saprophytic or pathogenic for animals and plants. Several plant diseases could be treated with good results (65). The culture media of the streptomycin producing *A. globisporus streptomycini* and of the biomycin producing *A. aureofaciens* contain 0.5 to 1.3 μ gm./ml. of vitamin $B_{12}$ when the culture is made in the presence of optimal concentrations of cobalt nitrate. Synthesis of the vitamin and of the antibiotics are simultaneous, and both substances can be produced during the same process (66).

A crystalline, antibiotic substance, identified as usnic acid, has been isolated from the lichen *Parmelia vagans;* it is active mainly on Gram-positive microbes, but also on *Hemophilus pertussis* and on some protists (67). Bactericidal effects of extracts from some plants (*Sanguisorba officinalis*, *Potentilla termentilla*, and from apples) on dysentery bacteria, even on sulfonamide-resistant strains, have been described (68).

Many publications are concerned with the action of bactericidal and bacteriostatic substances on bacteria of the enteric group, mainly *Shigella*. Various aspects of this problem have been investigated, such as cross resistance to various antibiotics and drugs; comparative resistance to antibiotics of strains possessing different degrees of virulence; formation of resistant strains and the persistence of their properties; action of antibiotics on the normal intestinal flora and existence of strains normally resistant to them; differences between a resistant strain, isolated from a patient; and a strain adapted *in vitro* to the same antibiotic, etc.

Under strict anaerobic conditions, the "microcid" does not show its bac-

teriostatic and bactericidal activity either on *Clostridium perfringens* or on *Staphylococcus aureus* (69). Phtyvazid-resistant strains of *M. tuberculosis* can be obtained *in vitro* or isolated from patients treated by this preparation. Such strains cultivated *in vitro* sometimes lose their resistance; whereas in man, resistant strains can be isolated even 4 to 12 months after the last treatment. Diminution or loss of virulence for experimental animals parallels the transformation into resistant strains (70).

Studies on the action of various antibiotic substances (mainly from actinomycetes) on bacteriophages and actinophages permitted the observation of a great variety of actions. Some of the phages were sensitive to most of the antibiotics, others, only to a few or to none of the antibiotics used (71).

The antibiotic activity of prodigiosin, the red pigment of *Serratia marcescens,* is correlated with its penetration into the cell of the bacterium or yeast attacked. Penetration of the pigment can be observed as well in the case of mixed cultures as in using pure pigment; it is progressive, and when the concentration is such that the attacked cell is either completely colored red or contains intensely colored drops or granulations, its multiplication is inhibited (72).

Investigations of Korotiaev on the mechanism of the antibacterial activity of chloramphenicol have permitted him to observe that the carbohydrate metabolism of *Streptococcus lactis* was not modified, and that the hexokinase, phosphorase, aldolase, and dehydrase activities of *E. coli* were not inhibited by it, but that the carbohydrate metabolism of the latter microbe was disrupted at the stage of pyruvic acid assimilation (73).

Series of publications in different journals have been devoted to clinical aspects of the use of antibiotics in different diseases, such as typhoid, dysentery, plague, anaerobic infections, brucellosis, tuberculosis, etc. In several cases it is recommended that specific serotherapy be used in conjunction with the antibiotics.

N. G. Kliueva and G. I. Roskin have published a monograph, "The problem of anti-tumor antibiotics," in Moscow, in 1957. It contains a description of the historical development of the experimentation in this field and the experimental and clinical results obtained by the authors with an extract from *Schizotrypanum cruzi.*

*Microbiology of soil, water, and oil.*—In a review on soil microbiology, Mishustin has envisaged the following questions: the methods of investigation of soil microbes; the soil microflora and the transformation which it provokes; and the relations between the microorganisms and the plants (74). The geographical distribution of antagonistic microorganisms has been reviewed by Gauze. He points out that the percentage of antagonistic fungi is higher in southern than in northern soils, and that those growing in the south have a larger antibiotic spectrum and a higher activity than those from the northern regions. The same is true for sporulating soil bacteria; thus, for example, *Bacillus mesentericus* is more common in the south than in the

north (75). Several articles have been published on the microbial flora of soils in different regions of the U.S.S.R.

Mekhtiev proposes the utilization of *Bacillus megaterium* and *B. cereus* as indicators in the evaluation of soils. The quantity of the former increases in the gradient between the soil of a forest and that of a cultivated field, whereas *B. cereus* is more abundant in noncultivated turf-podzols and its quantity in cultivated fields is very small (76). Soil bacteria which possess protopectinase activity, such as *C. polymixa, B. mesentericus, B. pumilus, B. mycoides, Pseudobacterium brunneum, P. fluorescens,* are abundant in the rhizosphere of fields, mainly on the second and third years of clover cultivation; and the quantity of *C. polymixa* may be used as an indication of the intensity of the protopectin degradation process in a soil (77). The active mineralization process in peaty soils is characterized by an intense multiplication of bacteria capable of developing on inorganic N-containing substances, by an increase in the quantity of sporulating bacteria and actinomycetes, and by the intensity of nitrification concomitant with the development of sporulating bacteria such as *B. megaterium* (78). Several other articles published in *Mikrobiologiya* concern analogous problems of soil microbiology.

The fungus *Lipomyces* (of the Endomycetaceae) has been regularly found in Yakoutia; it is a nitrogen-fixer and its distribution in various soils of this region has been described (79). A new and simplified method for the isolation of *Azotobacter* from the soil has been proposed by Beliakov; filter paper is used instead of the classical agar-gel technique (80). Even in the absence of *Leguminosae* clover nodule bacteria may survive for many years in the soil and preserve their virulence and N-fixation capacity (81).

Purple thiobacteria of Lake Belovod' grow in zones 13 to 14 meters deep, where they find $H_2S$ which they utilize. Using labelled $CO_2$, it could be established that 1.15 mg. $CO_2$/liter of the lake water was assimilated by these microorganisms in this zone (82). Labelled $S^{35}$ has permitted studies on the role of microorganisms in the formation of sulfur deposits in some wells, and on the reduction of sulfates in the artificial lake of Rybinsk. The generation time and the quantity of microbes in different parts of this lake have been investigated, as well as the number of microorganisms in various zones of the Black and Caspian seas at different periods of the year. According to Kriss (83), the microbial flora of the Arctic Ocean under the ice, although the temperature is constant, has seasonal variations, and during the winter dark period the number of organisms is greatly diminished. Various microbes and a yeast have been isolated from the water and the deposits of this Ocean (83).

Several articles have been published on the microflora of oil field waters at different depths and at different temperatures. In general, the number of microorganisms decreases with the depth. Rybakova has found various bacteria in oily waters at 3500 to 4200 meters; among these microorganisms she found denitrifiers and sulfate-reducers, as well as microbes which de-

compose proteins and ferment glucose (84). Kuznetsova and her collaborators admit that sulfate-reducing bacteria are actually growing in the deep waters in contact with the oil, using it for their nutrition. These investigators propose injecting formalin-containing water (100 mg./liter) in order to inhibit the formation of $H_2S$ (85).

Bacteria of the genus *Pseudomonas* can grow on media containing oil, and it has been observed that in anaerobiosis some bacteria may modify the quality of the oil: dimunition (5 per cent) of solid hydrocarbons and of the melting point of paraffins have been noted (86).

### Infectious Diseases

Among the articles published in the principal journals during the year under review, at least 130 to 150 should be mentioned under this heading. Lack of space obliges the present author to cite only a few of these publications. Statistically, the largest number of publications concern intestinal diseases (mainly dystentery), then rickettsial and viral diseases, and leptospirosis, but various other infectious diseases have also been studied. Epidemiology, persistence of infectious agents in natural conditions, isolation of variants with characterization of their pathogenic and bacteriologic properties, germ-carriers, and experimental infections in animals seem to be the most frequent subjects of investigation.

A series of three articles has been devoted by Diadichev to the theory of the epidemiological process. He envisaged the existence of relationships among the duration of an infection, the mechanism of its transmission, and the particular properties of its agent; and he emphasized the infections which are transmitted by arthropods (insects and ticks). The spread of an epidemic will depend upon such facts as the capacity of the infectious agent to live in the lower animal, and on the capacity of this animal to move about (flying insects). Thus, for example, epidemic diseases transmitted by ticks are characterized by their duration and by their restricted area of spreading. The cases of tularemia and plague are characteristic because they are infectious diseases of rodents and depend on ecological properties, but the epidemiology of the former, which is transmitted by ticks, is said to have different aspects than the latter which is transmitted by fleas (87). These last differences were submitted to criticism by Mikulin (88).

The use of different substances, capable of increasing the nonspecific resistance of the organism to infections, has been studied; and experiments performed on animals have shown that several pyrimidine derivatives produce useful effects, such as stimulation of phagocytosis or increase in antibody response, as well as stimulation of tissue regeneration and of hemopoiesis (89).

A series of articles on the afflictions of the nervous system during infectious diseases (such as encephalitis, brucellosis, influenza, tuberculosis) has appeared in Issue No. 3, Volume 57 (1957) of the *Zhurnal Nevropatologii i Psikhiatrii.*

Several authors have investigated the pathogenic mechanisms or effects of various bacterial toxins (diphtheria, tetanus, botulinus, gas gangrene). Adrenalectomy diminishes the resistance of rats to diphtheria toxin, whereas section of the principal nervous connections seems to have only a minor effect. These results are interpreted as a demonstration of the importance of humoral factors in the protective function of the adrenals (90). Injections of tetanus toxin into various sites or organs and into different parts of the brain of rabbits has lead to various observations, such as the following: for organs innervated by cranial nerves, injection directly into the organ has a more toxic effect than a subcutaneous injection of the same dose of toxin; the pathological manifestations are different when the toxin is introduced into different parts of the brain, etc. (91).

Comparative studies on the leucotoxic, hemolytic, and lecithinase activities of the toxins from *C. perfringens, C. oedematiens,* and *C. septicum* have been performed by Sirnik (92).

In connection with experimental studies on *Hemophilus pertussis,* investigations have been performed on *Brucella bronchiseptica* (many laboratory animals are carriers of this microbe). Antigenic properties and cultural aspects of it have been studied; when freshly isolated it is in phase I, but after preservation the strains pass into phase IV. The organisms in these phases possess somewhat different cultural and antigenic properties and show unequal antigenic relations with *H. pertussis* (93).

Differences among strains of staphylococci and streptococci isolated from various places have been related to the conditions in which they were growing. Thus, for example, staphylococci isolated in a meat processing plant were particularly adapted to producing hyaluronidase and proteases, whereas strains isolated from pastries frequently produced more coagulase (94). Selective localization of streptococci in certain organs, such as in joints, was observed after a number of passages of the strain through the brain or bone marrow of an animal. Lesions resembling certain aspects of human rheumatic diseases (endocarditis, interstitial myocarditis, etc.) have been produced in experimental animals (rabbits, rats), but the authors stress that the observed modifications do not entirely correspond to the characteristics of the human disease (95).

Zemskov, using the method of active anaphylactic shock with specific desensitization in experimental infections of mice by streptococci and shigellae, claims to have been able to observe the formation of alien antigens in the liver, the spleen, and the kidneys, whereas some of the normal antigens disappeared (96).

At least 20 publications appeared in the *Zhurnal Microbiologii Epidemiologii i Immunobiologii* on problems connected with dysenteric infections. The main subjects were: epidemiology; the persistence of bacteria in nature and in carriers and the importance of their control; the methods of discerning reinfections; description of local epidemics; the duration of the incubation period and the transformation of an acute into a chronic

infection; modification of the general intestinal microflora during dysenteric infections and after recovery from them; and experimental infections in different animals (mice, guinea pigs, kittens, apes). When seven apes, carriers of *S. flexneri,* were infected with the same microorganism, six showed clinical dysentery; whereas among four noncarriers submitted to the same infection, only one was ill (97).

Experimental infection of kittens with the same microorganism resulted, after an incubation period of five to six days, in clinical manifestations which appear to be very similar to human dysentery. Thus, this animal seems to be very useful in studies on the pathogenicity of dysentery (98). But particularly careful examination of the microflora of the animals used for experimentation is recommended, because they are often carriers of other pathogenic microbes (99). Experimental keratoconjunctivitis has been regularly obtained in guinea pigs when freshly isolated dysentery strains have been used. Local immunity against this microbe and, to a lesser degree, against other shigellae is observed in the infected eye; agglutinins appear in the blood, but the other eye is not immunized (100). Analogous results are described by Manolov, but he observed local resistance of the infected eye only after repeated infections. Vaccination of the animal with dysentery vaccines did not protect the eye against local infection (101).

Several articles describe the following: pathological cases from which different *Salmonella* have been isolated; the role of some *Shigella* variants in so-called "nonspecific" colitis; the different strains of *E. coli* and their associates which can be isolated from various sources and mainly from water; the importance of the strains 0111, 055, and 026 of *E. coli* in the toxic infections of the newborn; etc. A variant of *S. typhimurium* which does not produce gas was isolated from guinea pigs during an epidemic which showed a clinical aspect resembling pseudotuberculosis of rodents (102).

Modifications in the alkaline phosphatase level in the serum of typhoid and paratyphoid patients have been observed and a certain degree of parallelism with the severity of the state has been noted (103). Studies on nicotinic acid metabolism in typhoid patients treated with synthomycin have shown that the deficiency of this vitamin can explain some of the secondary clinical aspects of this infection (104). In normal rats submitted to various diets, the microflora show large modifications; for example, an increase of proteins (casein) in the diet provokes a diminution of lactic bacteria and an increase in *E. coli,* enterococci, and *Clostridium perfringens* in the feces (105).

Among publications on the epidemiology of brucellosis, an article of Kucherova may be cited. In order to establish the possible role of wild rats in the transmission of this disease this author has examined the blood and organs of 239 wild grey rats caught in endemic regions. None of the guinea pigs injected with this material had been infected, although experiments have proved that the wild rat can be infected with *Brucella* either by subcutaneous injections or by contact with infected rats, and that the infected

rats excrete living microbes in the urine and feces (106). Practical recommendations for the control of brucellosis in farm animals have been discussed (107).

The important role played by water in the propagation of tularemia has been stressed by Sil'chenko. When contaminated water was used for washing the pathologic manifestations in man were external; when the water was used for drinking, the symptoms were internal. In general, the water is infected by mice and particularly by water rats; most of the infections in man occur in autumn when these rodents migrate toward the towns (108). Distribution of *Pasteurella tularensis* in different organs of infected animals and their persistence in living animals, as well as in refrigerated meat or on grain and straw, have been studied. Morphological examinations of white rats infected with living microbes or injected with living or killed vaccines have been performed; the dispersion of the microbes in the organs of rats and the formation of typical granuloma have been described (109).

The pathogenicity of *Listerella monocytogenes* is extremely high for *Lagurus lagurus, Pall.;* 10 microbes kill them in three to five days, whereas 10 to 100 million are necessary to kill a white mouse or a *Microtus arvalis, Pall.* Pathological modifications produced in the mouse *Lagurus* by this microbe have been described, and the author recommends the use of this animal in experimental studies on listerellosis; their breeding in captivity is said to present no difficulties (110).

Use of *M. tuberculosis* labelled with $P^{32}$ has confirmed that their subcutaneous introduction into guinea pigs is very rapidly followed by their dispersion into different organs and tissues. Thirty minutes after the injection, the microbes have invaded the lymphatic nodes, spleen, bone marrow, lungs and are present in the blood. The quantity of microbes in different organs varies in time and shows maxima and minima (111). Experimental infection of chicks with different types of *M. tuberculosis* (human, bovine, and avian) has shown that when equal amounts (1 mg.) were used, the human type produced only a local reaction, and 30 days later the tuberculin reaction became positive. The bovine type produced some isolated nodules in internal organs, whereas the avian type gave a generalized tuberculosis. When human or bovine types are experimentally adapted to chicks, the lesions which they can induce are increased (112).

Bacterial tumors on plants have been the subject of several publications. A bacterial strain has been isolated which produces tumors on certain poplars; experimental infection could only be provoked in the spring. This microbe is probably identical with *Pseudomonas rimefaciens* isolated in Holland. Its serologic specificity has been studied (113). When tomato plants infected with *Agrobacterium tumefaciens* are in a stage of regeneration (for example, when a cutting is developing roots), the formation of the tumor is inhibited (114). Hybridizations of potato plants are said to favour the inhibition of the development of tumors by *Synchytrium endobioticum* (115).

Many different authors have published their investigations on rickettsial diseases, and particularly on Q-fever. Most of the articles concerned with this disease are assembled in Issue No. 6 of the *Zhurnal Mikrobiologii, Epidemiologii i Immunobiologii.* They describe the appearance of this disease in different regions of the U.S.S.R.; the transmission from sheep and cattle has been stressed. Most of the cases found were among workers in contact with meat and wool, but transmission by poultry has been also observed, and isolation of rickettsiae from egg shells has been described (116). The persistence of *Coxiella burneti* in meat and in milk submitted to various treatments has been studied, and it is stressed that normal means of pasteurization do not destroy this organism in the milk, but heating kills it even with only one minute at 100°C.

Other rickettsial diseases have also been studied, and articles on the epidemiologic aspects of initial and recurring types of typhus (117) and on the clinical and epidemiologic characteristics of murine (endemic) typhus (118) have been published. For the last 15 years in the town of Perm, a team of investigators in different specialties has studied different aspects of the Volhynian or paroxysmal fever, and a short account of their observations has been given. This disease is considered to have the same development as typhus, but because of less clear-cut symptoms it has not always been recognized (119). Pathogenic, clinical, and diagnostic aspects of polycyclic rickettsiosis, also called trench or five-day fever (rickettsiosis quintana) have been described. The author admits that this disease is considered to be very rare, mainly because no convenient means of diagnosis are available (120).

In 1951, a particular disease was observed in southeastern Turkemenia, and it was supposed that this disease could be of rickettsial origin. A special team of investigators was sent to this region, and some results of their study have been published. Ticks, *Hyaloma asiaticum,* were found to be carriers of rickettsiae which appeared to be pathogenic for guinea pigs and for white rats (the pathologic symptoms are described); animals recovering from the first infection were immunized against a second one. Chick embryos are also susceptible to this germ. No cross reactions with other rickettsiae could be observed, and the authors propose to consider this organism as a new species and to give it the name *Dermacentroxenus deserti Pavlovskyi,* Kulagin, 1953. The transmission through the *H. asiaticum* has been experimentally established in animals. The pathogenicity for man has not yet been clarified (121).

Epidemiological studies on mumps showed that the isolation period of patients can be shortened; nine days are said to be sufficient (122). The journal *Voprosy Virusologii* published several articles on influenza which were concerned with the epidemiology and prophylaxis of this disease, the symptomless carriers of viruses, and a morphologic study of the modifications induced in the respiratory organs of mice infected with influenza virus and submitted to x-rays (123). It has been shown, using experimental in-

fection of synantropic flies with poliomyelitis virus, that the virus may persist for 10 days in this fly and thus be transmitted by it (124). Toxic properties of four strains of viruses from the psittacosis-ornithosis group have been investigated on white mice; the toxic effect was neutralized by specific immune sera obtained from roosters (125). Studies on the cytotoxic effect of the tick-transmitted encephalitis virus on fibroblasts have been performed. Within 24 to 48 hr. after the introduction of the virus in the culture of fibroblasts, characteristic degeneration of the cells can be observed. Multiplication of the virus is optimal between the fourth and eighth days, but the virus is still active even after a month in the same culture medium. The activity of the virus is neutralized by an immune serum (126). Histologic modifications produced by Shope's virus have been described and the mechanisms of these modifications have been discussed by Ptokhov (127). Rous chicken sarcoma virus, when injected into young rats which have been inoculated with the same virus during their embryonic life, provokes a hemorrhagic disease, particularly severe if two injections have been given. The main lesions are subcutaneous, but sometimes internal organs (lungs) are also involved. These results, which seem to be an application of the phenomenon of acquired immunologic tolerance, demonstrate the possibility of transfering this chicken virus to a mammalian host (128).

In order to obtain arguments which would justify the hypothesis that schizophrenia is a virus disease, Malis has performed experiments using the technique of "agglutination of virus-coated microbes." He claims to have obtained positive results and that the blood and sometimes other materials (cerebrospinal fluid, duodenal liquid, nasal mucosa) from schizophrenic patients contain a specific antigen (129).

Some time ago, Morgulis and Shubladze envisaged the possibility of multiple sclerosis also being a virus-induced disease. With the idea that there may be an antigenic relationship between the supposed virus and rabies virus, Kolesnikov performed cutaneous tests with rabies "fixe" virus preparations in patients and claims to have observed a high percentage of positive results. Controls performed on normal persons and on patients suffering from other diseases gave negative results (130).

## Immunology

In an article summarizing previous discussions on immunologic problems, Vygodchikov proposed the following main subjects for future research and discussion: immunochemistry of antigens and their fate in the organism (use of labelled antigens in these studies); immunochemistry of antibodies and the morphologic basis of their formation; neurologic and neurohumoral regulations of the formation of antibodies and phagocytosis; influence of ionizing radiation on immunologic processes; and immunity by excretion (131).

Studies on the behavior of the inflammatory reactions in rabbits and mice after the first and second injections of living staphylococci or of a staphylo-

coccal vaccine have permitted Solov'ev to conclude that, at least under some conditions, no direct correlation can be established between antitoxin titers, phagocytic activity, and modification of the inflammatory reaction (132).

Some results on the diminished resistance to experimental infections in irradiated animals have been published, as well as observations on endogenous infections in such animals.

The phenomenon of acquired tolerance to antigenic substances has been confirmed using injections of horse serum into rat embryos (133). The same author has published a general review on this phenomenon and discussed its mechanism (134). As previously mentioned, this phenomenon seems to explain the possibility of transmitting chick sarcoma to mammals (128). The absence of immunologic reactivity in the foetus has been discussed in a general review on this question (135).

*Phagocytosis.* This process has been the subject of many publications. Modifications in phagocytic activity during different periods of a disease, under the influence of certain drugs, and during x-ray irradiation have been described. Some authors insist on the role of the nervous system in promoting or inhibiting phagocytosis. Thus, for example, observations on the modifications of phagocytic activity corresponding to different periods in scarlet fever and diphtheria were said to be parallel to different states of the sympathetic or the vegetative nervous system (136). This problem has been reviewed by Puchkov who admits, on the basis of numerous personal experiments and observations reported in the literature, that substances liberated by the sympathetic nerve endings stimulate phagocytosis, whereas the parasympathetic substances inhibit it (137).

The action of cortisone and deoxycorticosterone on phagocytosis has been investigated. Prolonged administration of cortisone to rabbits did not suppress phagocytosis of *E. coli* by macrophages, but these cells did not lyse the absorbed microbial bodies. Deoxycorticosterone had, on the contrary, a stimulating effect (138). Analogous results have been obtained on mice infected with *S. typhosa* and receiving cortisone; phagocytosis persisted, but the absorbed bacteria were not destroyed (139). Phagocytosis of *Clostridium perfringens* in the peritoneum of immunized and nonimmunized rabbits and guinea pigs in the presence or absence of the toxin has been studied. Immunization seems to have no influence on phagocytosis, but the toxin diminishes it markedly (140). The usefulness of the method employing phagocytosis for the detection of small amounts of botulinus toxin, already mentioned in a previous review, has been confirmed.

Cutaneous tests have been used as a means of observing the immunologic reactiveness of persons living in northern regions; notable diminution of it has been seen during the polar night (141).

The problem of antibody formation has been studied by many investigators, and several publications are concerned with such questions as modifications in the blood or in the distribution of serum proteins during immunization or infection; the action of antibiotics, of carbon tetrachloride poison-

ing, and of irradiation on immunization or on antibody formation; and the use of labelled substances in immunologic research, etc. For example, $S^{35}$-labelled methionine and liver proteins have been used in studies on the rate of formation of antibodies and other serum proteins in rabbits (142); $C^{14}$-glycine has been utilized in order to follow the rate of formation of two different antibodies. From this last study, it has been concluded that even very intense formation of one antibody does not inhibit the rate of synthesis of another antibody, or of nonspecific $\gamma$-globulins, and that the formation of antibodies takes place only a short time before their appearance in the circulation; whereas for 2 to 3 days after the injection of the antigen, no measurable synthesis of antibodies or of any kind of antibody-precursor could be detected (143).

Differences in the formation of antiphage antibodies in cows, calves, sheep, and rabbits—depending on the route of phage injection or ingestion—have been observed, and the necessity of taking into account the formation of antiphage antibodies, when phage is utilized as a therapeutic measure, has been stressed (144).

According to Kozlovskaia, the antibodies in a young animal are the result of a combined active and passive immunization, because she found circulating antibodies in offspring of actively immunized females 45 to 80 days after birth. In the case of passively immunized females and in passively immunized offspring, the persistence of circulating antibodies in the young animal was restricted to 8 to 14 days (145).

Continuing their studies on the transfer of the capacity to form antibodies, Shtertsl & Grubeshova[3] have injected nucleoprotein-rich fractions (containing nuclei and mitochondria) from spleens of adult rabbits immunized with *S. schottmuelleri* into 5-day-old rabbits, and observed the appearance of antibodies in the serum of the young rabbits (146).

Daily oral administration (40 mg./kg.) of pentoxyle to rabbits during immunization against *B. abortus* has resulted in higher agglutin titers than observed in controls, and Kalinin proposes to use this substance in combination with antibiotics in the treatment of brucellosis (147). Novoembikhin, i.e., 2-chlorethyl-2-chlorpropylamine, may influence the complement titer and, depending on the time of its injection as compared to the antigen administation, stimulates or inhibits the formation of antibodies (148, 149).

[3] It seems useful to stress here the consequences of the application of the rules for the transcription into Latin letters of names written in Slavonic. These two authors normally publish in Czechoslovakia and write their names: Sterzl and Hrubesova, which become Shtertsl and Grubeshova when transcribed from their Russian publications. The same is true in the case of Shorin (63), who wrote his name Chorine, when he worked in France and published in French. The original names of Russian authors of foreign origin have also become completely transformed. Thus, for example, we write the name Zhak, which, based on its pronunciation, should have probably been written Jacques, and we write Zil'ber for a name which was certainly originally Silber.

Rabbits have been immunized separately with the toxin and with atoxic proteins extracted from *Clostridium perfringens*, and it could be shown that protection against a severe test (injection of spores) arises only from the antitoxins; antibodies against nontoxic antigens were devoid of any protective activity (150).

Several publications concerned the preparation, fractionation, titration, and protective activity of hyperimmune sera against diphtheria, tetanus, cholera, rabies, influenza, encephalitis, etc. Efimova claims that the "physiological state" or the "nervous type" of a horse used for the production of antitoxic sera is to be considered in order to choose the schedule of antigen injections and obtain desirable titers (151). A stimulation in antitoxin production is said to be obtained in horses when they were freed from parasites (helminths) (152).

Use of purified γ-globulin fraction from horse antirabies immune sera is recommended in combination with vaccination in cases of dangerous bites (head, face, neck, hands, etc.) by rabid animals and particularly by wolves (153, 154).

Guinea pigs sensitized with preparations of horse thrombin showed anaphylactic shock when injected with therapeutic horse immune sera, and the danger of injections of normal horse sera (for example in protein therapy) which can sensitize for a possibly necessary injection of therapeutic immune serum, is stressed (155).

A book, *The Cytotoxins in Contemporary Medicine*, has been published by the Ukrainian state medical editions in Kiev, U.S.S.R. It contains 40 articles on the preparation, the use, and the mechanism of action of cytotoxic sera, mainly of the antireticular cytotoxic serum (Bogomoletz serum).

Studies on tumor antigens have been pursued using complement-fixation tests and active anaphylaxis with desensitization by extracts of corresponding normal tissues. The limits of this last method according to Gardash'ian are of the order of few micrograms (156). Correlation between the sensitizing and the shocking doses of antigen have also been envisaged, and it was found that when the sensitizing dose is diminished, the shocking dose must be increased (157).

Comparative studies on three different tumors (Ehrlich's adenocarcinoma, Crooker's sarcoma, and an acridine-induced sarcoma), obtained in a pure breed of mice, have been made using complement-fixation, and antigenic differences among the three tumors and also between these tumors and normal tissues have been observed (158). Using the same method and immune sera of rabbits, goats, and horses, extracts of various human tumors have been compared, and the results obtained are interpreted as showing that some tumors possess the same, while others, possess different antigens (159, 160).

Rabbits in which a regression of a Brown-Pearce tumor was obtained by ultrasonic irradiation, become resistant to a second implantation of the

same tumor (161). Guinea pigs sensitized with Ehrlich's ascitic cells are not completely desensitized with cells previously irradiated with x-rays; a modification of the specific antigens seems to explain this result (162).

Several articles are concerned with problems related to bacterial toxins. Some of the main subjects of these publications may be cited: preparation of potent diphtheria toxins using stab cultures; antigenic and allergenic properties of toxins; results obtained in vaccinations performed with purified and adsorbed diphtheria, tetanus, and gas gangrene toxoids; combined vaccinations with various toxoids and with bacterial vaccines. Several authors confirm the superiority of results obtained with adsorbed toxoids. Intramuscular injection of tetanus toxoid is said to induce an earlier appearance and a higher titer of antitoxin in man (163). Injection of tetanus toxin into the vagus nerve of rabbits has not only attacked motile functions, but also produced hemorrhagic lesions in lungs (164). A pure preparation of histolyticum toxin was obtained: it was electrophoretically homogeneous and contained 19 different amino acids and no carbohydrate (165). Streptococcal scarlatina toxin treated with 0.5 per cent formol for 30 days at 40°C. lost its toxicity, but its antigenic properties were lowered (166). Experiments on cats have shown that staphylococcal enterotoxin is particularly toxic for the heart; even very dilute solutions stop the contraction of an isolated heart (167).

Vaccinations against intestinal diseases were the subject of many publications, mostly in the *Zhurnal Mikrobiologii Epidemiologii i Immunobiologii.* Isolation of conveniently attenuated strains, different means for the preparation of killed bacteria (use of alcohol or phytoncides), studies on the antigenic properties of various vaccines and of purified antigen preparations, use of purified endotoxin in cutaneous tests, etc., have been described. In addition, the influence of the number of injections, the intervals between them (168), and the site where they are made on the immunization of experimental animals has been studied (169). In children submitted to vaccine therapy and in experiments on white mice, differences in the agglutinin titers and in phagocytosis were observed when equal doses of vaccines of *S. sonnei* and *S. flexneri* were compared; higher agglutinin titers, but poorer protection of mice, were observed with *S. flexneri* vaccine (170). Various antigenic fractions and their subfractions obtained by different methods from *Shigella* cultivated on synthetic media were analyzed and their antigenic properties compared. No direct correlation could be established between their chemical composition and their immunogenic properties; however, the most antigenic fractions are said to be complexes formed by polysaccharides and proteins (171). A study on antigens prepared by Boivin's method from *S. sonnei* gave analogous results. In many rabbits, discrepancies between the time of maximum titers of precipitins and agglutinins have been noted (172).

Increase in the immunogenic properties and stabilization of plague

vaccine (living) are said to be achieved by the passage of the strain through guinea pigs (173). Experiments performed on rabbits and mice with cholera vaccines administered at various intervals showed that the bactericidal and the agglutinin titers are not always correlated (the ratios are inverted in different periods of immunization), and the authors conclude that the agglutinin titer should not be considered as a measure of the state of immunization (174).

Among publications concerning BCG vaccination, such questions have been considered as the preservation of dried vaccine, the dosage of the vaccine to be used in the vaccination of guinea pigs, and the desensitization produced when numerous vaccinations with large amounts of the vaccine have been performed. The modifications in the allergic cutaneous test and the increase of resistance to a virulent strain, as a consequence of such treatments, have been described (175).

Many authors have published the results of their investigations on vaccination or revaccination against brucellosis with live vaccine. In most cases, satisfactory results are described, particularly in humans [see, for example, a review by Vershilova (176)]; but according to Rotov, attempts to cure spontaneously infected animals with the vaccine prepared from strain No. 19 have not been successful (177). The fundamental immunological mechanism in brucellosis seems to be phagocytosis, and its increase in immunized animals has been studied (178). Two types of antigenic complexes have been obtained from *Brucella,* one of which contains nucleic acids; the protein components of these two complexes show chemical and immunochemical differences (179).

Extracts from *Brucella* and from *Pasteurella tularensis* obtained by ultrasonic disintegration were proposed for allergic cutaneous tests. Several articles are concerned with such questions as combined vaccination against brucellosis and tularemia, the preparation of a dried tularemic vaccine, the duration of immunity in vaccinated persons (more than four years), the use of allergic tests before revaccination, etc. The characteristic properties of the attenuated strain used in vaccination against tularemia and its dissociation have been described (180).

Vaccinations against virus-induced diseases, methods of preparation of vaccines, and their titration and use have been described by different authors. Thus, in the case of rabies vaccine, the following aspects have been envisaged: purification of the vaccine from the brain tissue, increase in the activity of rabies vaccine, neurological complications following this vaccination, etc. Use of ACTH in animal experimentation on encephalomyelitis has permitted an amelioration of the clinical symptoms (181).

A review on the results obtained with a live antiinfluenza vaccine has been published by Smorodintsev & Zhdanov (182). The immunogenic properties of this vaccine and the possible reasons for influenza cases among vaccinated persons have been described (183). The method of preparation

and the properties of a dried, attenuated live vaccine against mumps, as well as its use in intradermal vaccination in children, have been published (184).

More than 40 articles were devoted to the description of various microbiologic or serologic methods and their applications for diagnostic purposes. Most of the authors used classical or slightly modified methods, but some publications describe either completely new or at least not commonly used techniques; few of them may be cited. The concentration of a bacterial suspension can be measured by using its characteristic luminescence in ultraviolet light (185). Use of specific antibodies rendered fluorescent by coupling with fluorescein (Coon's method) has been tried for the diagnosis of various intestinal microbes, but the results are judged to be insufficient (186). Extremely small amounts of *Shigella* (less than one cell per ml.) could be detected by using a method based on the increase in the titer of specific phages (187). This method has also been applied for typhoid baccili, and is said to permit rapid diagnosis. Another method which shortens the time necessary for a bacteriologic typhoid diagnosis is the use of lysates of microbe-feeders in the culture media (188). A new method for quantitative estimation of antibodies has been described by Gurvich. The antigen is fixed by diazotation on filter paper, and the quantity of antibody which such a filter paper absorbs from a serum is measured by a colorimetric method after washing, drying, and combination with a dye. Even nonprecipitating antibodies can be estimated by this procedure (189). The hemagglutination reaction produced by vaccinia virus can be inhibited by different dyes, among which acrichin (an acridine derivative) was the most active; its acts on the erythrocyte. Hemagglutination by the influenza virus was not inhibited by these dyes (190).

### The Role of the Nervous System in the Pathogenesis of Infectious Diseases and in Immunology

This problem is still one of the main points in official research programs, and several authors have published articles discussing its theoretical aspects. Large differences of opinion exist, and these articles often contain severe mutual criticism. Issue No. 3 of *Trudy Instituta vysshei nervnoi deiatelnosti, seriia patofisiologicheskaia* (1957) contains some 20 articles devoted to this problem. A kind of summary is given in the first of them by Ivanov-Smolenskii: "Some problems of the pathophysiology of central nervous activity and of the experimental therapy of its disturbance, in connection with studies on the cephalotropic influence of intoxications and infections." It would be impossible to summarize here the results of this contribution, which was based on a very large number of experiments performed on animals with conditioned reflexes, and supplemented by histologic examinations of their brains. Among other established facts, it may be interesting to point out that an action on the central nervous system has been observed even for those toxic substances of bacterial origin which are not generally considered as possess-

ing one. The main conclusions are as follows: in intoxications as well as in infections, the evolution, character, and extent of the disturbances in the activity of the central nervous system depend upon the type of nervous activity of the animal, and upon the particular character of a given toxin, its dose, and its manner of penetration into the central nervous system. Many data have been obtained on "sleeping therapy" for various infections in rats. When appropriate conditions were chosen, the disturbances in the activity of the central nervous system induced by the infection could be healed much more quickly when the animal was subjected to medicinally-induced sleep for a certain time. Among the articles, following this introduction by Ivanov-Smolenskii, seven are concerned with experimental studies on the superior nervous activity of animals during intoxications or infections, eight describe the results obtained in the experimental therapy of the disturbances observed, and four relate to the histopathology of the central nervous system during infections and intoxications. The same issue also contains an article by Khozak on the disturbances in the activity of the central nervous system of guinea pigs caused by experimental anaphylactic reactions. In most cases, inhibition of the reflexes has been noted. Normal activity of the nervous system seems to be reestablished sooner when sleep is induced by medication.

Several other journals have published articles by other authors on the same problem. A few of them have been mentioned previously in connection with other studies. According to Mitina (191), an artificial excitation of the central nervous system by strychnine induces a greater resistance of the animal to tetanus toxin. Aliev has studied the action of *Vibrion septique* (*Clostridium septicum*) toxin on rats and has admitted that pathologic excitation of the central nervous system must not be overlooked (192). The role of chemoreceptors of the intestinal tract in the mechanism of intoxication in dysentery has been described (193). In experimental tuberculosis in guinea pigs, modifications of conditioned reflexes have been observed (194).

In different psychoses caused by infectious diseases, the immunobiologic reactivity of the organism shows variations which are said to be correlated with the evolution of the psychosis (195). Observations recorded on tetanus antitoxin-producing animals have permitted Evseev to classify such animals according to different groups, depending upon their "nervous type." The time required to obtain a maximum titer of antitoxin is said to be a function of this "type" (196).

The role of reflexes in antibody response has again been stressed (197, 198), particularly as an answer to a previous criticism by A. D. Ado. In order to demonstrate the participation of the nerves, Gordienko and his collaborators performed experiments in which they introduced the antigen (a bacterial suspension) into isolated carotid sinuses or spleens of dogs. The authors claim that antibody formation was observed when such isolated spleens retained their nervous connections in the organism, and that suppres-

sion of these connections resulted in the absence of antibody formation (199). Analogous results were described when cocaine was used to inhibit the nervous receptors of an isolated sinus (200).

## LITERATURE CITED


1. Adams, S., *Federation Proc.*, **16**, 716–20 (1957)
2. Rakhno, P. K., and Tokhver, V. I., *Doklady Akad. Nauk S. S. S. R.*, **112** (1), 144–45 (1957)
3. Chernomordik, A. B., Kuritsina, N. M., and Kapul, N. M., *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, Suppl. 1956, 9 (1957)
4. Drazin, R. S., *Voprosy Virusol.*, **2** (1), 29–33 (1957)
5. Kantarovich, R. A., *Voprosy Virusol.*, **2** (4), 208–10 (1957)
6. Gershenzon, S. M., *Doklady Akad. Nauk S. S. S. R.*, **113** (5), 1161–62 (1957)
7. Protsenko, A. E., *Byull. Glavn. Botan. Sada*, **27**, 98–107 (1957)
8. Fedotina, V. L., *Izvest. Akad. Nauk S. S. S. R., Ser. Biol.* **22** (1), 114–16 (1957)
9. Peremitina, L. D., *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (7), 56–61 (1957)
10. Mamatsashvili, E. G., *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (9), 8–11 (1957)
11. Melkumova, T. A., *Mikrobiologiya*, **26** (3), 313–14 (1957)
12. Draganov, K. I., *Biofizika*, **2** (3), 348–50 (1957)
13. Gershenzon, S. M., *Byull. Moskov. Obshchestva Ispytatelei Prirody. Otdel. Biol.*, **61** (6), 99–101 (1956)
14. Sukhov, K. S., and Kapitsa, O. S., *Doklady Akad. Nauk S. S. S. R.*, **113** (6), 1366–68 (1957)
15. Vasilenko, A. G., *Mikrobiologiya*, **26** (2), 160–66 (1957)
16. Loginova, L. G., *Mikrobiologiya*, **26** (4), 485–97 (1957)
17. Sluvko, A. L., *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (8), 26–29 (1957)
18. Kondrat'eva, V. F., *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (8), 39–41 (1957)
19. Nabokov, I. S., *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (1), 20–23 (1957)
20. Moroz, A. F., *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (5), 115–20 (1957)
21. Bezborodov, A. M., Kashkin, K. P., and Iamshchikov, V. P., *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (3), 108–14 (1957)
22. Martyniuk, I. V., *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (3), 131 (1957)
23. Pelevina, M. V., *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (6), 101–4 (1957)
24. Pshenichnov, A. V., Sheveleva, O. N., and Noskova, E. G., *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (7), 11–14 (1957)
25. Sheinker, A. P., and El'piner, I. E., *Biofizika*, **2** (3), 354–57 (1957)
26. Filippovich, E. M., *Veterinariya*, **34** (4), 45–49 (1957)
27. Sukhov, K. S., *Agrobiologiya*, No. 1, 36–53 (1957)
28. Vainberg, B. G., Cherviakova, K. I., Brutman, E. I., and Choporova, M. I., *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (3), 91–100 (1957)
29. Smirnovskaia, A. S., *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (2), 16–19 (1957)
30. Veisfeiler, I. K., Inogamov, A. B., and Engalycheva, A. M., *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (2), 8–12 (1957)

31. Levashev, V. S., *Antibiotiki*, **2** (2), 12–18 (1957)
32. Levashev, V. S., *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (8), 29–35 (1957)
33. Konosh, O. V., *Voprosy Virusol.*, **2** (2), 104–10 (1957)
34. Gubarev, E. M., and Galaev, I. V., *Ukraïn. Biokhim. Zhur.*, **29** (2), 196–98 (1957)
35. Belozerskiĭ, A. N., Zaitseva, G. N., Gavrilova, L. P., and Mineeva, L. V., *Mikrobiologiya*, **26** (4), 409–17 (1957)
36. Spirin, A. S., Belozerskiĭ, A. N., Vaniushin, B. F., and Shugaeva, N. V., *Biokhimiya*, **22** (4), 744–54 (1957)
37. Bitkova, A. N., and Semcheva, N. S., *Biokhimiya*, **22** (3), 495–500 (1957)
38. Fedorov, M. V., *Mikrobiologiya*, **26** (2), 232–42 (1957)
39. Seliber, L. G., *Mikrobiologiya*, **26** (4), 426–31 (1957)
40. Kalinenko, V. O., *Mikrobiologiya*, **26** (2), 148–53 (1957)
41. Elin, V. L., and Vasiurenko, K. I., *Mikrobiol. Zhur. Akad. Nauk Ukr. R. S. R., Inst. Mikrobiol. im. D. K. Zabolotnogo*, **19** (3), 11–13 (1957)
42. Lozinov, A. B., and Ermachenko, V. A., *Mikrobiologiya*, **26** (2), 154–59 (1957)
43. Ruban, E. L., Rylkin, S. S., and Pokrovskaia, N. V., *Mikrobiologiya*, **26** (3), 257–62 (1957)
44. Maksimova, I. V., *Doklady Akad. Nauk S. S. S. R.*, **112** (4), 766–768 (1957)
45. Rashba, E. I., *Mikrobiologiya*, **26** (3), 277–284 (1957)
46. Domaradskii, I. V., *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (6), 7–12 (1957)
47. Oparin, A. I., Gelman, N. S., and Zhukova, I. G., *Biokhimiya*, **22** (2–1), 399–403 (1957)
48. Kolchins'ka, I. D., *Mikrobiol. Zhur. Akad. Nauk Ukr. R. S. R., Inst. Mikrobiol. im. D. K. Zabolotnogo*, **19** (1), 3–16 (1957)
49. Petrosian, A. P., *Izvest. Akad. Nauk Armyan. S. S. S. R., Biol. i Sel'skokhoz. Nauki*, **10** (4), 95–105 (1957)
50. Ryzhkov, V. L., *Izvest. Akad. Nauk S. S. S. R. Ser. Biol.*, **22** (1), 41–54 (1957)
51. Lomteva, M. N., *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (8), 105–7 (1957)
52. Zubok, L. B., and Blagoveshchenskii, V. A., *Biokhimiya*, **22** (4), 695–98 (1957)
53. Shemanova, G. F., Blagoveshchenskii, V. A., *Biokhimiya*, **22** (5) 799–803 (1957)
54. Mordashev, S. R., and Lu Ju-Shan, *Biokhimiya*, **22** (1–2), 369–74 (1957)
55. Lu Ju-Shan, *Mikrobiologiya*, **26** (3), 271–76 (1957)
56. Gubarev, E. M., and Galaev, I. V., *Biokhimiya*, **22** (3), 441–44 (1957)
57. Zakharova, I. I., *Mikrobiol. Zhur. Akad. Nauk Ukr. R. S. R., Inst. Mikrobiol. im. D. K. Zabolotongo*, **19** (1), 17–39 (1957)
58. Zavarzin, G. A., *Doklady Akad. Nauk S. S. S. R.*, **113** (6), 1361–62 (1957)
59. Sokurova, E. N., and Meisel', M. N., *Biofizika*, **2** (4), 483–86 (1957)
60. Tumanian, M. A., *Vestnik Rentgenol. i Radiol.*, **32** (1), 14–18 (1957)
61. Sokurova, E. N., *Mikrobiologiya*, **26** (4), 444–49 (1957)
62. Andonov, P. S., *Voprosy Virusol.*, **2** (4), 228–34 (1957)
63. Shorin, V. A., *et al., Antibiotiki*, **2** (5), 44–49 (1957)
64. D'iakov, S. I., *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (9), 80–81 (1957)
65. Krasil'nikov, N. A., *et al., Mikrobiologiya*, **26** (4), 418–25 (1957)

66. Surikova, E. I., and Popova, L. A., *Mikrobiologiya*, **26** (4), 432–37 (1957)
67. Zatulovskii, B. G., *Mikrobiol. Zhur. Akad. Nauk Ukr. R. S. R., Inst. Mikrobiol. im. D. K. Zabolotnogo*, **19** (2), 34–37 (1957)
68. Bogoiavlenskaia, N. L., *Farmakol. i Toksikol.*, **20** (2), 54 (1957)
69. Sakhnovskaia, G. K., and Shablovskaia, E. A., *Mikrobiol. Zhur. Akad. Nauk Ukr. R. S. R., Inst. Mikrobiol. im. D. K. Zabolotnogo*, **19** (2), 38–42 (1957)
70. Ginzburg, T. S., *Problemy Tuberk.*, **35** (1), 81–86 (1957)
71. Krasil'nikov, N. A., and Kafanova, N. D., *Antibiotiki*, **2** (1), 5–10 (1957)
72. Boriu, S. I., *Mikrobiologiya*, **26** (4), 464–67 (1957)
73. Korotiaev, A. I., *Mikrobiologiya*, **26** (4), 450–57 (1957)
74. Mishustin, E. N., *Pochvovedenie*, **11**, 62–79 (1957)
75. Gauze, G. F., *Uspekhi Sovremennoĭ Biol.*, **43** (1), 46–54 (1957)
76. Mekhtiev, S. I., *Mikrobiologiya*, **26** (1), 75–77 (1957)
77. Lambina, V. A., *Mikrobiologiya*, **26** (1), 66–74 (1957)
78. Zimenko, T. G., *Izvest. Akad. Nauk S. S. S. R., Ser. Biol.*, **2**, 234–40 (1957)
79. Mazilkin, I. A., *Mikrobiologiya*, **26** (4), 477–80 (1957)
80. Beliakov, E. V., *Mikrobiologiya*, **26** (2), 186–88 (1957)
81. Fedorov, M. V., and Egorova, S. V., *Izvest. Timiryazev. Sel' skokhoz. Akad.*, **2**, 98–110 (1957)
82. Lialikova, N. N., *Mikrobiologiya*, **26** (1), 92–98 (1957)
83. Kriss, A. E., *Doklady Akad. Nauk S. S. S. R.*, **114** (1), 199–202 (1957)
84. Rybakova, S. G., *Doklady Akad. Nauk S. S. S. R.*, **115** (4), 813–15 (1957)
85. Kuznetsova, V. A., *et al., Mikrobiologiya*, **26** (3), 330–37 (1957)
86. Kolesnik, Z. A., and Shmonova, N. I., *Doklady Akad. Nauk S. S. S. R.*, **115** (6), 1197–99 (1957)
87. Diadichev, N. R., *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (1), 5–10; (2), 44–50; (3), 8–14 (1957)
88. Mikulin, M. A., *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (10), 142–44 (1957)
89. Lazarev, N. V., *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (10), 41–47 (1957)
90. Iurkov, I. A., *Arkh. Patol.*, **19** (3), 51–56 (1957)
91. Katsitadze, V. A., *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (8), 111–15 (1957)
92. Sirnik, I. O., *Mikrobiol. Zhur. Akad. Nauk Ukr. R. S. R., Inst. Mikrobiol. im. D. K. Zabolotnogo*, **19** (2), 14–19 (1957)
93. Osipova, P. V., *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (10), 137–42 (1957)
94. Knizhnikov, V. A., and Kasatkina, I. L., *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (1), 80–84 (1957)
95. Borodinok, N. A., and Beletskaia, L. V., *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (10), 66–70 (1957)
96. Zemskov, M. V., *Byull. Eksptl. Biol. Med.*, **43** (2), 66–70 (1957)
97. Dzhikidze, E. K., *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (4), 58–62 (1957)
98. Budylina, V. V., *et al., Byull. Eksptl. Biol. Med.*, **43** (2), 70–75 (1957)
99. Khomik, S. R., *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (4), 62–65 (1957)
100. Gekker, V. D., Belaia, I. A., and Mel'nik, E. G., *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (7), 110–14 (1957)

101. Manolov, D. G., *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (10), 94–98 (1957)
102. Emel'ianova, O. S., *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (9), 63–64 (1957)
103. Budzhe, M. M., and Bliuger, A. F., *Terap. Arkh.*, **29** (9), 58–64 (1957)
104. Borisova, M. A., *Terap. Arkh.*, **29** (2), 70–76 (1957)
105. Askalonov, S. P., Dobrier, I. B., and Shvaiko, V. A., *Voprosy Pitaniya*, **16** (2), 58–62 (1957)
106. Kucherova, N. T., *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (9), 25–29 (1957)
107. Lokhov, M. G., and Gurevich, R. M., *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (9), 16–21 (1957)
108. Sil'chenko, V. S., *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (6), 15–21 (1957)
109. Mashkov, A. V., and Taranenko, A. F., *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (8), 122–25 (1957)
110. Dunaeva, T. N., *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (9), 51–55 (1957)
111. Arkhipova, O. P., and Kozulitsina, T. I., *Problemy Tuberk.*, **35** (2), 96–103 (1957)
112. Kokurichev, P. I., *Doklady Akad. Sel' skokhoz. Nauk*, **22** (3), 45–48 (1957)
113. Chumaevskaia, M. A., *Doklady Akad. Sel' skokhoz. Nauk*, **22** (3), 40–44 (1957)
114. Terekhova, N. A., *Fiziol. Rasteniĭ, Akad. Nauk S. S. S. R.*, **4** (1), 72–76 (1957)
115. Gedz', S. M., *Doklady Vsesoyuz. Akad. Sel' skokhoz. Nauk, im. V. I. Lenina*, **22** (9), 28–30 (1957)
116. Zhmaeva, Z. M., and Pchelkina, A. A., *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (3), 39–41 (1957)
117. Pastukhova, G. M., *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (9), 91–95 (1957)
118. Imamaliev, S. A., *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (3), 47–53 (1957)
119. Pshenichnov, A. V., *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (10), 19–24 (1957)
120. Gal'perin, E. A., *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (6), 62–66 (1957)
121. Kulagin, S. M., *et al., Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (7), 114–21 (1957)
122. Ganzburg, S. E., *et al., Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (2), 38–39 (1957)
123. Smorodintsev, A., *Voprosy Virusol.*, **2** (5), 290–96 (1957)
124. Levkovich, E. N., and Sukhova, M. N., *Med. Parazitol. Parazitar-. Bolezni*, **28** (3), 343–47 (1957)
125. Semenov, B. F., *Voprosy Virusol.*, **2** (1), 25–29 (1957)
126. Zasukhina, G. D., and Levkovich, E. N., *Voprosy, Virusol.*, **2** (4), 234–38 (1957)
127. Ptokhov, M. P., *Byull. Eksptl. Biol. Med.*, **44** (7), 93–96 (1957)
128. Zil'ber, S. A., and Kriukova, I. N., *Voprosy Virusol.*, **2** (4), 239–43 (1957)

129. Malis, G. I., *Zhur. Nervopatol. i Psikhiatrii,* **57** (1), 82–86 (1957)
130. Kolesnikov, G. F., *Zhur. Nervopatol. i Psikhiatrii,* **57** (3), 317–22 (1957)
131. Vygodchikov, G. V., *Zhur. Mikrobiol. Epidemiol. Immunobiol.,* **28** (5), 3–9 (1957)
132. Solov'ev, V. N., *Zhur. Mikrobiol. Epidemiol. Immunobiol.,* Suppl. 1956, 1 (1957)
133. Kriukova, I. N., *Byull. Eksptl. Biol. Med.,* **43** (4), 78–79 (1957)
134. Kriukova, I. N., *Uspekhi Sovr. Biol.,* **44** (1), 127–41 (1957)
135. Aliverdiev, A. A., *Uspekhi Sovremennoĭ Biol.,* **43** (1), 97–103 (1957)
136. Brainina, E. S., and Kapustiak, S. I., *Zhur. Mikrobiol. Epidemiol. Immunobiol.,* **28** (2), 23–24 (1957)
137. Puchkov, N. V., *Uspekhi, Sovremennoĭ Biol.,* **43** (2), 165–79 (1957)
138. Ertuganova, Z. A., and Ageeva, L. S., *Byull. Eksptl. Biol. Med.,* **43** (1), 74–77 (1957)
139. Forshter, K. K., *Zhur. Mikrobiol. Epidemiol. Immunobiol.,* **28** (4), 139–46 (1957)
140. Shneerson, A. N., *Zhur. Mikrobiol. Epidemiol. Immunobiol.,* **28** (4), 83–86 (1957)
141. Piriatinski, L. B., *Zhur. Mikrobiol. Epidemiol. Immunobiol.,* **28** (3), 65–66 (1957)
142. Uchitel', I. I., and Konikova, A. S., *Byull. Eksptl. Biol. Med.,* **44** (7), 85–89 (1957)
143. Gurvich, A. E., and Smirnova, N. P., *Biokhimiya,* **22** (4), 626–35 (1957)
144. Kvesitadze, I. F., and Mikhailova, I. F., *Zhur. Mikrobiol. Epidemiol. Immunobiol.,* **28** (1), 99–104 (1957)
145. Kozlovskaia, L. A., *Voprosy Virusol.,* **2** (4), 220–24 (1957)
146. Shtertsl, I., and Grubeshova, M., *Zhur. Mikrobiol. Epidemiol. Immunobiol.,* **28** (4), 39–46 (1957)
147. Kalinin, B. I., *Zhur. Mikrobiol. Epidemiol. Immunobiol.,* **28** (4), 51–54 (1957)
148. Lutsenko, T. A., Markitantova, A. E., and Timokhina, M. I., *Byull. Eksptl. Biol. Med.,* **44** (9), 89–93 (1957)
149. Shamaeva, E. M., and Pankova, S. S., *Zhur. Mikrobiol. Epidemiol. Immunobiol.,* **28** (4), 25–30 (1957)
150. Vygodchikov, G. V., *et al., Zhur. Mikrobiol. Epidemiol. Immunobiol.,* **28** (10), 120–25 (1957)
151. Efimova, N. P., *Zhur. Mikrobiol. Epidemiol. Immunobiol.,* **28** (1), 94–99 (1957)
152. Khomiakov, A. M., Mendelevich, M. M., and Gonin, S. L., *Zhur. Mikrobiol. Epidemiol. Immunobiol.,* **28** (1), 110–14 (1957)
153. Selimov, M. A., Kovalevskii, M. F., and Semenova, E. V., *Zhur. Mikrobiol. Epidemiol. Immunobiol.,* **28** (9), 35–41 (1957)
154. Mikhalenko, Z. V., *Voprosy Virusol.,* **2** (1), 56 (1957)
155. Chertkova, F. A., Didenko, S. I., and Ushakova, A. A., *Zhur. Mikrobiol. Epidemiol. Immunobiol.,* **28** (8), 107–10 (1957)
156. Gardash'ian, A. M., *Zhur. Microbiol. Epidemiol. Immunobiol.,* **28** (1), 71–74 (1957)
157. Ioffe, V. I., and Kopytovskaia, L. P., *Byull. Eksptl. Biol. Med.,* **44** (7), 82–84 (1957)
158. Airaper'ian, G. P., *Byull. Eksptl. Biol. Med.,* **44** (11), 85–92 (1957)
159. Kosiakov, P. N., and Kuznetsova, N. I., *Byull. Eksptl. Biol Med.,* **43** (6), 49–53 (1957)

160. Korosteleva, V. S., *Byull. Eksptl. Biol. Med.*, **44** (8), 87–93 (1957
161. Dimitrieva, N. P., *Byull. Eksptl. Biol. Med.*, **43** (6), 60–62 (1957)
162. Maiskii, I. N., and Suvorova, G. V., *Byull. Eksptl. Biol. Med.*, **44** (9), 94–96 (1957)
163. Pletsityi, D. F., *et al.*, *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (4), 3–10 (1957)
164. Katsitadze, V. A., *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (6), 79–83 (1957)
165. Ispolatovskaia, M. V., *Biokhimiya*, **22** (6), 1000–3 (1957)
166. Pervachenko, S. V., *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (9), 81–82 (1957)
167. Stolmakova, A. I., *Byull. Eksptl. Biol. Med.*, **44** (11), 57–62 (1957)
168. Lipkin, M. E., *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (5), 25–30 (1957); **28** (7), 45–51 (1957)
169. Batiuk, I. F., *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (3), 61–64 (1957)
170. Zherikova, A. D., *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (4), 69–75 (1957)
171. Dubrovskaia, I. I., *et al.*, *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (4), 126–33 (1957)
172. Diachenko, S. S., Bernasovs'ka, E. P., and Anchevs'ka, M. S., *Mikrobiol. Zhur. Akad. Nauk Ukr. R. S. R., Inst. Mikobiol. im. D. K. Zabolotnogo*, **19** (1), 44–53 (1957)
173. Korobkova, E. I., *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (7), 64–68 (1957)
174. Khristov, A. C., and Gylybov, K. C., *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (4), 73–76 (1957)
175. Nakhimson, L. I., and Gnevysheva, E. F., *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (7), 23–28 (1957)
176. Vershilova, P. A., *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (10), 24–29 (1957)
177. Rotov, I. V., *Doklady Vsesoyuz. Akad. Sel' skokhoz. Nauk im. V. I. Lenina*, **22** (6), 41–43 (1957 )
178. Chernysheva, M. I., *Doklady Vsesoyuz. Akad. Sel' skokhoz. im. V. I. Lenina*, **22** (9), 11–15 (1957)
179. Dubrovskaia, I. I., *Biokhimiya*, **22** (5), 924–28 (1957)
180. Emel'ianova, O. S., *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (8), 125–29 (1957)
181. Saper, E., and Gausmanova, I., *Zhur. Nevropatol. i Psikiatrii*, **57** (2), 172–78 (1957)
182. Smorodintsev, A. A., and Zhdanov, V. M., *Voprosy Virusol.*, **2** (2), 67–72 (1957)
183. Zakstel'skaia, L. I., Iakhno, M. A., and Efimova, V. A., *Voprosy Virusol.*, **2** (4), 213–19 (1957)
184. Kliachko, N. S., and Maslenikova, L. K., *Voprosy Virusol.*, **2** (1), 13–17 (1957)
185. Vasilov, S. I., Nechaev, V. V., and Radionova, L. N., *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (2), 59–63 (1957
186. Dashkevich, I. O., and Mikhailov, I. F., *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (6), 66–73 (1957)

187. Gol'dfarb, D. M., *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (1), 26–29 (1957)
188. Karpuzidi, K. S., and Drozhevkina, M. S., *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (6), 92–97 (1957)
189. Gurvich, A. E., *Biokhimiya*, **22** (6), 1028–34 (1957)
190. Zeitlenok, N. A., Pille, E. R., and Koniush, O. V., *Voprosy Virusol.*, **2** (5), 273–78 (1957)
191. Mitina, T. V., *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, Suppl. 1956, 5 (1957)
192. Aliev, A. N., *Doklady Akad. Nauk S. S. S. R.*, **113** (3), 713–75 (1957); *Doklady Akad. Nauk S. S. S. R.*, **113** (4), 220–22 (1957)
193. Miagkaia, I. P., *Byull. Eksptl. Biol. Med.*, **44** (11), 52–57 (1957)
194. Chertkova, M. A., *Zhur. Vysscheĭ Nervnoĭ Deyateľnosti im I. P. Pavlova*, **7** (2), 285–90 (1957)
195. Kasatkina, G. V., and Chikaleva, L. V., *Zhur. Nevropatol. i Psikhiatrii*, **57** (9), 1068–75 (1957)
196. Evseev, V. A., *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (7), 90–94 (1957)
197. Pletsityi, D. F., *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (1), 129–36 (1957)
198. Gordienko, A. N., *Zhur. Mikrobiol. Epidemiol. Immunobiol.*, **28** (1), 136–41 (1957)
199. Gordienko, A. N., *et al.*, *Byull. Eksptl. Biol. Med.* **43** (4), 80–82 (1957)
200. Gordienko, A. N., *et al.*, *Byull. Eksptl. Biol. Med.*, **44** (11), 72–75 (1957)

# ANTIBIOTICS IN THE CONTROL OF PLANT DISEASES[1]

By William J. Zaumeyer
*Crops Research Division, Agricultural Research Service, U. S. Department of Agriculture, Beltsville, Maryland*

The use of antibiotics is a comparatively new method of plant disease control, but antibiosis, or antagonistic phenomena among microorganisms, and its relation to plant diseases have been recognized for more than 75 years. Gliotoxin, the first antibiotic to be used for plant disease control, was isolated and purified by a plant pathologist even before the discovery of penicillin. In 1932 Weindling (1) noted the antagonism of *Gliocladium fimbriatum* to a number of fungi. In 1934 he studied (2) a lethal principle produced by this organism and found that it was responsible for the parasitic action of *G. fimbriatum* on *Rhizoctonia solani* and other soil fungi. Two years later Weindling & Emerson (3) isolated in crystalline form the substance known as gliotoxin, thus becoming the first to isolate an antibiotic in a pure state. The next report of the control of a plant disease by an antibiotic was by Brown & Boyle (4, 5) in 1944. They observed that a crude culture filtrate of *Penicillium notatum* was effective against crown gall, caused by *Agrobacterium tumefaciens*. No *in vivo* evidence of the antibiotic effect of the filtrate on the pathogen was reported, and later studies showed that the effect was largely on the gall tissue. In 1945 Brian & Hemming (6) showed that gliotoxin reduced infection of covered smut of barley, bunt of wheat, and helminthosporium leaf spot of oats. In 1946 Brown & Heep (7) reported the eradication of *Xanthomonas pruni* from cankers on plum twigs. It was not until 1952, however, that an antibiotic was used under field conditions for the control of plant disease. Zaumeyer *et al.* (8) showed that beans were protected from infection by the halo blight organism (*Pseudomonas phaseolicola*) with a streptomycin spray, and Ark (9) reported good control of pear blight (*Erwinia amylovora*) with a streptomycin dust.

Previously, most of the investigations consisted of screening plant pathogens *in vitro* to determine their sensitivity to antibiotics. Although screening tests *in vitro* demonstrated the effectiveness of a number of antibiotics against many pathogens, tests *in vivo* frequently failed to corroborate them.

This review deals primarily with the control of diseases of plants, under greenhouse and field conditions, by means of the antibiotics which are commonly available. Certain compounds which have been studied only a little and are unavailable to growers are not discussed. It is likely that among the large number of papers published in this field, some important ones were over-

[1] The survey of the literature pertaining to this review was concluded in December, 1957.

looked. Previous reviews were very helpful in the preparation of this paper (10 to 15).

## DISEASES OF FRUITS AND NUTS

### Apple and Pear

*Fire blight.*—Fire blight of apples and pears, caused by *Erwinia amylovora,* was the first bacterial disease of an orchard tree to be effectively controlled with an antibiotic under commercial conditions. The first report on such control was by Murneek (16) of Missouri in 1952. Spraying apple trees once with aqueous solutions of thiolutin or streptomycin during blossoming protected them somewhat from blight infection. Later, very promising results were obtained with various streptomycin-formulation sprays by Goodman (17, 18, 19) in Missouri; Young & Winter (20) in Ohio; Heuberger & Poulos (21) in Delaware; Ark (9, 22), Ark & Scott (23), and Dunegan *et al.* (24) in California; Kienholz (25) in Oregon; Clayton (26) in North Carolina; Kirby (27) in Pennsylvania; Mills (28) in New York; and Miller (29) in Connecticut. These sprays drastically reduced blight infections on pears and apples wherever they were used. In 1953 Goodman (18, 19) in Missouri obtained 100 per cent control of fire blight of apples by using streptomycin, either in combination with oxytetracycline (Terramycin) or alone, at concentrations of 100 to 500 p.p.m. Complete control resulted when six applications of the antibiotic sprays were made at 4-day intervals, beginning at 30 to 50 per cent of full bloom. In 1954 Goodman (19), using Agri-mycin at concentrations of 50 and 100 p.p.m., found little difference in blight control between a 4- and a 3-spray schedule. A 4-spray schedule with Agri-mycin at a concentration of 25 p.p.m. also gave a high degree of protection. Even though secondary blight was severe in several of the plots, trees receiving four or three applications at 100 p.p.m. or 50 p.p.m. remained relatively free of infection for as long as 30 days after the first primary infections were observed. Young & Winter (20), in 1953, obtained 97 per cent control of blossom blight and 100 per cent control of twig blight of apples with three applications of streptomycin at 120 p.p.m.

Goodman (30) in 1955 found that streptomycin was relatively ineffective against late or mid-season forms of twig blight infection which often develop two to five weeks after full bloom.

Ark (9) in 1953, using crude streptomycin as a dust to control pear blight, found that it was less effective than copper-lime dust, but that it did not cause the fruit-russeting produced by the latter. In 1955 he (22, 31) reported that dusts containing 500 and 1000 p.p.m. of streytomycin blended with pyrophyllite gave good control of fire blight of pear, comparable with that given by copper-lime dusts.

Dunegan *et al.* (24), in tests conducted in California in 1954, demonstrated satisfactory control of blight with spray dosages of Agri-mycin as low as 30 p.p.m. applied five times during blossoming. When concentrations

of 60 and 100 p.p.m., respectively, were used, the disease was almost eliminated.

### Cherry

*Leaf spot.*—Hamilton & Szkolnik (32) reported that actidione (Actidione), sprayed on foliage at the rate of 2 p.p.m. after an infection period of 96 hr., controlled cherry leaf spot (*Coccomyces hiemalis*). Cation (33) controlled the disease by applying this antibiotic at a concentration of 1 p.p.m. twice before harvest and at 2 p.p.m. once after harvest. Effective control was also obtained with this antibiotic by several other investigators (34, 35, 36). Gilmer (37) showed that a mixture of actidione (2 p.p.m.) and sulfur (2 to 5 lb./100 gal. of water) afforded excellent control of both cherry leaf spot and powdery mildew of cherries when applied four times at 14-day intervals. Actidione alone, at the same concentration, gave excellent control of leaf spot but not of powdery mildew.

Prescott, Emerson & Ford (38) noted that when cherries had been given a preharvest treatment with actidione bioassays of the extracts detected as little as 0.04 p.p.m. of the antibiotic in the fruit.

Hamilton, Szkolnik & Sondheimer (39) demonstrated that the isomer acetate and semicarbazone derivatives of actidione in greenhouse tests protected against cherry leaf spot at 0.05 p.p.m. and gave good eradicative control at 1 p.p.m. when applied as a foliage spray four days after inoculation. The oxime derivative used at 60 p.p.m. also provided protection.

*Bacterial canker.*—In England, Crosse & Bennett (40) reduced infection of bacterial canker of cherry (*Pseudomonas syringae*) with five spray applications of streptomycin at a concentration of 200 p.p.m. The antibiotic was also highly effective against the leaf spot phase of the disease.

### Peach

*Bacterial canker.*—Dye & Dye (41) showed that under greenhouse conditions seedling peach trees could be protected from bacterial canker (*Pseudomonas syringae*) with streptomycin sulfate and dihydrostreptomycin sulfate sprays at a concentration of 100 p.p.m. Later Dye (42) reported that the antibiotic was also effective in preventing the disease in seedlings under field conditions in New Zealand, and that the causal organism had shown no signs of resistance to streptomycin.

*Bacterial spot.*—In 1946 Brown & Heep (7) reported the elimination of *Xanthomonas pruni* from cankers on plum twigs with streptomycin. Goodman & Shepard (43) in 1956 showed that Agri-mycin sprays appreciably reduced leaf spotting and defoliation caused by this organism.

Daines (44) in New Jersey reported that a combination of captan (2 lb./100 gal. of water) and streptomycin (150 p.p.m.) showed promise in controlling bacterial spot. The sprays were applied on a 7- to 10-day schedule after considerable fruit spot had appeared. Daines & Gray (45) found

that the addition of glycerol increased streptomycin absorption by peach leaves and hence increased the effectiveness of the antibiotic against infection by the bacterial spot organism. Old leaves showed considerably more absorbed streptomycin than did young ones.

Dunegan, Wilson & Morris (46, 47) reported that injection of oxytetracline into trees gave protection against the disease but that this treatment produced chlorosis of the foliage.

*Brown rot.*—Alcorn & Ark (48) found that candicidin dip protected peach fruits against infection by the brown rot fungus (*Sclerotinia fructicola*). Their tests also indicated that it also protected *Prunus* seedlings against the disease and that it was as effective as captan. Szkolnik & Hamilton (49) reported similar results with nystatin (Mycostatin), fungichromin, and oligomycin used as post-harvest dips at concentrations of 100 and 200 p.p.m. Di Marco & Davis (50) likewise found nystatin effective. Ogawa & Vergara (51) noted that actidione as a spray (1 p.p.m.) gave excellent protection against the disease.

### Miscellaneous Fruits

Strong & Klomparens (52) showed that a single application of actidione at 100 p.p.m. to the galls on red cedar prevented the germination of the teliospores of *Gymnosporangium juniperi-virginiana* and *G. globosum.*

Ogawa & Vergara (51) noted that sprays containing 2 p.p.m. of actidione protected grapes from powdery mildew, caused by *Uncinula necator,* and also eradicated the disease.

Ark & Alcorn (53) found candicidin effective as a protectant against infection of *Ribes* by the uredial stage of *Cronartium ribicola.*

In Russia, Mirzabekian (54), immersing apricot and peach cuttings in a 1 to 1000 streptomycin solution for 24 hours protected them from infection by *Bacterium armeniaca.*

In 1944 Brown & Boyle (4, 5, 55) injected crude penicillin into crown galls produced by *Agrobacterium tumefaciens* on *Bryophyllum* and showed that the growth of the tumors was checked. They determined later that the effect of the antibiotic was largely on the galled host tissue and not on the pathogen. In 1948 Brown (56) showed that the living gall cells as well as the causal organism were killed by penicillin and streptomycin. In the same year Hampton (57) also reported that these two antibiotics cured many different kinds of fruits affected with crown gall. Blanchard (58) found that fewer and smaller galls developed on tomato plants grown in a mineral nutrient solution containing chlortetracycline (Aureomycin) following needle inoculations with *A. tumefaciens* than on similar plants grown on mineral solution alone.

Klemmer, Riker & Allen (59) noted that 2 p.p.m. of oxytetracycline, 5 p.p.m. of chlortetracycline, and 4 p.p.m. of chloramphenicol introduced into tomato plants through root absorption inhibited the development of crown

gall. Less effective were streptomycin, patulin (clavacin), polymyxin, penicillin, and tyrothricin. Gall inhibition by oxytetracycline, chlortetracycline, and chloramphenicol was reported to be caused by the effects of the antibiotics on the gall cells. Deep (60) reported that preplanting treatments of roots of 2-year-old cherry trees for 15 min. in solutions containing 400 p.p.m. of oxytetracycline or in 200 p.p.m. for 1 hr. and later dipping in a bacterial suspension of the organism appreciably reduced gall formation. Treatment in 400 p.p.m. of Agri-mycin for 1 hr. also proved effective. Zuckerman (61) found that gall formation on high bush cranberries was not suppressed when galled roots were treated with Agri-mycin 100, Agristrep, Phytomycin, or actidione at weekly intervals during the period from dormancy through bud break. Ark & Sibray (62) obtained some control of crown gall of roses by dipping cuttings in a streptomycin solution containing 500 p.p.m. of the antibiotic. Unsatisfactory control resulted from dusts containing up to 2000 p.p.m. of streptomycin. A 100 p.p.m.-tetracycline dip gave 67 per cent control in Manetti rose and only 40 per cent in Paul's Scarlet rose.

### Walnut

*Blight.*—Ark (63) and Ark & Charles (64) reported the control of walnut blight, caused by *Xanthomonas juglandis,* with two sprays of 10 p.p.m. streptomycin sulfate. The applications were made at pre- and postbloom stages, and the control was about equal to that obtained with copper sprays.

## DISEASES OF VEGETABLES

### Bean and Lima Bean

*Bacterial blights.*—Smith (65) in 1949 and Hildreth & Starr (66) in Wyoming in 1950 found streptomycin effective in treating bean seed infected with the halo blight organism (*Pseudomonas phaseolicola*). In greenhouse tests, 1-hr. treatment of the seeds in a 10,000 p.p.m.-streptomycin solution gave complete control. In field tests, however, Starr *et al.* (67) noted some infection. In greenhouse and field tests, Klinkowski, Kohler & Schrodtler (68) in Germany also found that soaking infected seed in a crude streptomycin solution containing 100 p.p.m. of the antibiotic reduced infection.

In 1952, Mitchell, Zaumeyer & Anderson (69) demonstrated that under greenhouse conditions bean seedlings could be protected from halo blight by the application of a minute amount of streptomycin sulfate to the stems of the plants before inoculation with the causal bacterium. The stems absorbed the antibiotic and translocated it upward into the primary leaves in sufficient amounts to protect the plants from infection.

Field studies (8, 70) showed that when bean plants that had been artificially inoculated with the halo blight organism were sprayed three times at weekly intervals with 1000 p.p.m. of streptomycin, they were completely

protected from infection. Later tests showed that control was equally good when the concentration of streptomycin was only 250 p.p.m. Dye (71) in 1956 reported that five weekly applications of a 75 p.p.m.-streptomycin solution reduced halo blight infection in bean fields in New Zealand.

Field tests in 1954 (72) demonstrated that the halo blight organism was largely eradicated by an Agri-mycin spray containing 200 p.p.m. of streptomycin. Woolliams (73) in 1957 reported similar results.

Although streptomycin protected beans from infection by the common bean blight organism (*Xanthomonas phaseoli*) in the greenhouse (69), field results did not confirm these findings. Field plots thoroughly sprayed two or three times with streptomycin showed no common blight infection early in the season, but diseased plants were observed later. It was demonstrated that streptomycin would not control the disease if it were applied to beans after *X. phaseoli* had gained entrance into the plants and before symptoms appeared.

Marlatt (74) was unable to control common blight on Pinto beans in New Mexico with four applications of streptomycin sulfate or Agri-mycin spray at strengths up to 1000 p.p.m. of streptomycin. Infected seed treated for 15 min. in a 1 to 500 aqueous solution of streptomycin sulfate produced plants with as high an incidence of infection as the controls.

Andersen's (75) results, however, showed that beans could be protected against both common blight and fuscous blight (*X. phaseoli* var. *fuscans*) by seven weekly applications of a 200 p.p.m.-streptomycin spray.

Gray (76) reported that the addition of glycerol to streptomycin sprays caused a marked increase in effectiveness against *X. phaseoli* in greenhouse tests. A spray containing 50 p.p.m. of streptomycin sulfate and 1 per cent glycerol was more effective than one containing 200 p.p.m. of streptomycin alone. One application of a solution containing 200 p.p.m. of streptomycin and 1 per cent glycerol gave almost complete control of the disease. This high degree of control was still evident two weeks after the spray was applied. The increase caused by glycerol in effectiveness against the disease was correlated with the increase in absorption of streptomycin by the leaves. Andersen (75) also found that addition of glycerol increased the effectiveness of streptomycin against common and fuscous blights at the 200 p.p.m.-level, but not at the 400 p.p.m.-level. Silber & Kainski (77) observed that a 200 p.p.m.-streptomycin solution containing 0.5 per cent glycerol was not as effective in controlling halo blight as a 300-p.p.m. solution of streptomycin alone.

*Powdery mildew.*—In 1948 Felber & Hamner (78) showed that actidione spray at a concentration of 1 to 5 p.p.m. controlled powdery mildew (*Erysiphe polygoni*) of bean under greenhouse conditions. This was the first reported control of a fungus disease by an antibiotic. Zaumeyer (79) found that sprays of anisomycin at a concentration of 50 p.p.m. and griseofulvin at 100 p.p.m. protected bean seedlings from powdery mildew. The disease was eradicated by anisomycin at 50 p.p.m. and by griseofulvin at 200 p.p.m.

*Rust.*—Alcorn & Ark (80) reported that single spray applications of 1 to 5000 candicidin up to three days before inoculation were effective in reducing bean rust (*Uromyces phaseoli typica*). Zaumeyer & Wester (81) noted that sprays containing 100 p.p.m. of anisomycin or oligomycin or 440 p.p.m. of griseofulvin also protected beans from rust. Ziffer *et al.* (82) controlled the disease in the greenhouse with two new polypeptide antibiotics, Phytoactin and Phytostreptin.

*Anthracnose.*—In Germany, Grummer & Mach (83) reported that bean seeds infected with anthracnose (*Colletotrichum lindemuthianum*) were almost completely freed from infection when immersed in a 0.05 per cent streptomycin solution for 24 hr. Zaumeyer & Wester (81) showed that spray applications of nystatin at 450 p.p.m. concentration and of oligomycin at 100 p.p.m. protected bean seedlings from anthracnose.

*Downy mildew of lima beans.*—In greenhouse tests at Beltsville, Maryland, excellent control of downy mildew of lima beans, caused by *Phytophthora phaseoli,* was obtained with commercial streptomycin formulations. Zaumeyer & Wester (84, 85) reported that sprays of Agri-mycin 100, Agristrep and Phytomycin containing 100 p.p.m. of streptomycin gave excellent protective control of the disease. An Agri-mycin 500 spray containing 25 p.p.m. of streptomycin and 25 p.p.m. of neutral copper gave considerable better protection against the disease than did neutral copper or Agri-mycin 100, each at 25 p.p.m. The control action was synergistic.

From field tests in Delaware in 1956, Crossan *et al.* (86) reported that two applications of streptomycin, plus 1.5 lb. of Tri-basic copper per 100 gal. of water or streptomycin, plus 1 gal. of glycerol per 100 gal. of water were not nearly so effective in controlling downy mildew of lima beans as were maneb at the rate of 2 lb. per 100 gal. of water or Tri-basic copper at 4 lb. per 100 gal.

Zaumeyer & Wester (81) found that sprays containing 100 p.p.m. of anisomycin, 300 p.p.m. of nystatin, 50 p.p.m. of oligomycin, or 300 p.p.m. of filipin also protected lima beans from downy mildew infection in the greenhouse.

*Stem anthracnose.*—Lima bean seedlings were protected from stem anthracnose, caused by *Colletotrichum truncatum,* by spray applications of oligomycin at a concentration of 50 p.p.m. (81).

### Celery

*Bacterial blight.*—Cox (87) reported that 5 applications either of Agri-mycin at 300 and 600 p.p.m. of streptomycin or of Copper A at 4 lb. per 100 gal. gave high degrees of control of bacterial blight (*Pseudomonas apii*), but that the former appeared to be more effective. When copper was used, the disease gradually built up, whereas when Agri-mycin was used, the severity of the disease diminished. When these two materials were combined and applied as a spray, the mixture appeared to be more effective than either material alone, but it was not significantly better than Agri-mycin alone.

### Crucifers

*Downy mildew of broccoli.*—From field tests in New York in 1955, Natti *et al.* (88, 89) reported that four applications at weekly intervals of Agrimycin 100 as a foliage spray at the rate of 1 lb. in 100 gal. of water per acre showed promising results in controlling downy mildew (*Peronospora parasitica*). In 1957 Natti (90) found that four applications of an Agristrep spray at the rate of 3 lb. of the antibiotic in 100 gal. per acre provided excellent control of the disease as long as 27 days after the final spray application. In greenhouse tests, anisomycin, nystatin, streptomycin, and thiolutin sprays were also effective.

*Black rot of rutabaga.*—Sutton & Bell (91) reported in 1954 that treating rutabaga seeds in aqueous solutions of chlortetracycline provided effective control of seed-borne infection of the black rot organism (*Xanthomonas campestris*). Greenhouse soil tests showed that seed treatment increased germination. At 1 to 2500 and 1 to 1000 dilutions the organism was eliminated; in the untreated check, 76 per cent of the seedlings were infected.

Similar results were observed in field trials. Mature roots of plants from chlortetracycline-treated seed showed no evidence of infection, while 30 per cent of those from untreated seed showed infection.

### Cucumber

*Angular leaf spot.*—By soaking cucumber seed infected with *Pseudomonas lachrymans* in a 100 p.p.m.-solution of streptomycin, Ark (92) eliminated the organism. Doolittle & Beecher (93) found that Agri-mycin and Agristrep at 400 p.p.m. of streptomycin protected field-grown cucumber plants from infection.

*Wilt.*—Williams & Lockwood (94) reported that in greenhouse tests sprays containing 100 p.p.m. of streptomycin or neomycin or 500 p.p.m. of oxytetracycline or penicillin applied 24 hr. before inoculation of cucumber with the bacterial wilt organism (*Erwinia tracheiphila*) appreciably reduced disease incidence. Applications of 500 p.p.m. of streptomycin or neomycin gave complete control. In field tests sprays containing 100 p.p.m. streptomycin or 200 p.p.m. oxytetracycline also reduced the incidence of the disease. Plots sprayed with 500 p.p.m. streptomycin produced yields 25 per cent higher than the untreated plots.

*Downy mildew.*—Ark & Thompson (95) obtained satisfactory control of downy mildew of cucumber (*Pseudoperonospora cubensis*) in greenhouse tests when the plants were sprayed with a number of antibiotics either prior to or following inoculation with swarm spores of the causal organism. Sprays containing 500 p.p.m. of amphomycin, endomycin, gramicidin, nystatin, and oxytetracycline; 100 p.p.m. of patulin, polymyxin, and tyrothricin and a streptomycin dust containing 1000 p.p.m. of the antibiotic were effective.

Coe (96) reported control of the disease with seven applications, at four-

or five-day intervals, of Agri-mycin at 200 p.p.m. of streptomycin and 100 p.p.m. of streptomycin plus 2 and 4 lb. of Tri-basic copper per 100 gal. of water, respectively.

*Scab.*—Under field conditions promising results were obtained by de Zeeuw & Vaughn (97) in the control of cucumber scab, caused by *Cladosporium cucumerinum*, with actidione sprays at a concentration of 10 p.p.m. Seven applications at seven- to ten-day intervals were made.

### Onion

*Downey mildew.*—Nelson (98) reported significant yield increases in onions infected with downy mildew, caused by *Peronospora destructor*, when they were sprayed with a two per cent actidione-sulfur mixture.

### Pea

*Ascochyta blight.*—Dekker (99) observed that soaking pea seed infected with *Ascochyta pisi* in a solution of rimocidin at a concentration of 100 p.p.m. for 18 hr. gave a very high degree of control of the fungus.

### Potato

*Seed-piece decays.*—Bonde (100) in greenhouse tests and Bonde & de Souza (101) in greenhouse and field tests found that seed-piece decays causd by *Erwinia atroseptica* and *Pseudomonas fluorescens* could frequently be controlled by immersing infected, freshly cut potato seed pieces in a water solution of streptomycin sulfate or in one containing this antibiotic and oxytetracycline hydrochloride. Treatment for 10 to 30 min. in a solution containing 25 p.p.m. of either of these preparations practically eliminated the diseases. Of the untreated, infected seed pieces, 80 to 100 per cent decayed. Oxytetracycline hydrochloride alone did not control the diseases. No blackleg-diseased plants were produced from seed pieces treated with streptomycin sulfate or the combination of the two antibiotics. Two to five per cent of the plants from infected, untreated seed pieces were infected with blackleg.

In field experimentation, Bonde (102), Epps (103), and Robinson & Hurst (104) found that treating potato seed pieces in Agri-mycin solutions containing 100 p.p.m. of streptomycin produced similar results. Bonde (102) noted that an instant dip was as effective as a 30-min. soak. Treatment increased the emergence of the potatoes, reduced the percentage with blackleg, improved the color, size, and vigor of the plants, and increased the yield.

Although materials containing streptomycin sulfate were found by Bonde & Malcolmson (105) to be very effective in controlling bacterial seed-piece decay, seed pieces treated with streptomycin solutions showed an increased amount of fusarium and phoma rots and the causal fungi grew more rapidly in streptomycin-treated than in untreated potato slices.

Good control of fungus and bacterial decays of potato seed pieces was

obtained with Dithane (Nabam), Phygon, and captan following treatment with streptomycin (106). Rimocidin sulfate alone or in combination with Agri-mycin 100 gave good control of fungus rots (106).

*Ring rot.*—Van Schaack (107), Perkins (108), Bonde (109), and Logsdon (110) reported that streptomycin as a dip reduced ring rot (*Corynebacterium sepedonicum*) in potatoes.

*Late blight.*—Bonde (111) observed that when the ends of potato stems were immersed for 72 hr. in a solution containing 100 p.p.m. of oxytetracycline, infection by the late blight fungus (*Phytophthora infestans*) was delayed and sporangial production on the leaves was sparse in contrast to that on the check plants. The fungus was controlled with a spray containing 100 p.p.m. of thiolutin. Soil applications with it as well as with twelve other antibiotics were ineffective. Hodgson (112) reported that in Canada Agri-mycin, Agristrep, and crude dihydrostreptomycin applied as foliage sprays at a concentration of 200 p.p.m. of the antibiotics gave good control of the disease.

### Spinach

*Soft rot.*—Smith (113, 114), using streptomycin at a concentration of 1000 p.p.m. as a preharvest spray and as a postharvest dip, reduced bacterial soft-rot decay of spinach caused by *Erwinia carotovora* and *E. atroseptica* so that the treated leaves showed virtually no decay after two days' storage at 70°F.

### Tomato and Pepper

*Bacterial spot.*—Conover (115) in 1954 reported good control of bacterial spot (*Xanthomonas vesicatoria*) in tomato plant beds with five applications of an Agri-mycin spray containing 200 p.p.m. of streptomycin. Of the treated seedlings, 74 per cent were disease-free and only 0.4 per cent were severely infected. In the untreated beds 12.8 per cent of the plants were disease-free and 34.8 per cent were severely diseased. In the treated beds, 95 per cent of the plants were suitable for transplanting as compared with only 27 per cent in the untreated.

Coe (116) reported control of bacterial spot in Florida after it was generally established in the field and the plants were setting fruit. Five applications of Agri-mycin at 400 p.p.m. of streptomycin and three at 200 p.p.m. were made at approximately four- to five-day intervals. The treated plots showed a 42 per cent greater yield, a 28 per cent greater average fruit size, and an 11 per cent greater number of fruits than the untreated plots.

Conover (117) also reported very satisfactory field control of the disease with seven applications of Agri-mycin at 200 p.p.m. of streptomycin. In one test there were 67 per cent less fruit infection, a 12 per cent greater average fruit size, a 50 per cent greater yield of disease-free fruit, and a 25 per cent greater total yield on the treated plots than on the check plots. In a second test the sprays caused 85 per cent and 72 per cent reduction in fruit infection in areas of the field set with healthy and disease-infected transplants, respectively.

Conover's (115) results in controlling bacterial spot on pepper seedlings were similar to those on tomato seedlings. Five applications of Agri-mycin at 200 p.p.m. of streptomycin effectively controlled the disease. Cox, Comegys & Heuberger (118) reported that three applications of streptomycin at a concentration of 500 p.p.m. eradicated the disease in severely infected field-grown peppers. Crossan & Krupka (119) found that Agri-mycin reduces infection of pepper leaves when used at frequent intervals at concentrations of 250 p.p.m. or higher but does not eradicate the causal organism from leaf lesions when used at concentrations as high as 500 p.p.m.

In tests during the 1954-55 season in Florida, Cox (120, 121) found that weekly applications of streptomycin formulations gave commercial control of the disease, and that the effectiveness increased up to 400 p.p.m. Yield was as much as 50 per cent more than that of the checks. Neutral coppers also effectively controlled the disease. They were compatible with Agri-mycin, and when they were used in combination with it there appeared to be considerable additive effect. In later tests Cox (122) found that 200 and 400 p.p.m. of the antibiotic plus copper were more effective. Altman & Davis (123) noted that one application of a 200-p.p.m. streptomycin nitrate spray significantly reduced the number of leaf spot lesions.

*Early blight.*—Brian *et al.* (124) in 1951 and Stubbs (125) in 1952 reported that griseofulvin applied to the roots of tomato plants grown in sand culture protected the foliage against early blight (*Alternaria solani*). Stubbs (125), applying the antibiotic to the foliage, concluded that leaf application was less effective than root absorption in the systemic control of the disease. Davis & Rothrock (126) in greenhouse tests effectively reduced early blight of tomato with a griseofulvin spray. Their results showed that the effect of the antibiotic persisted in the tissues for at least seven days.

Endomycin (127), toximycin (128), and antimycin A (129) applied as foliage sprays were also reported to control the disease in greenhouse tests. Ziffer *et al.* (82) demonstrated that Phytoactin and Phytostreptin effectively controlled the disease in the greenhouse.

*Late blight.*—Tomato seedlings were effectively protected against infection by *Phytophthora infestans* in the greenhouse with sprays containing 100 p.p.m. of streptomycin (130).

*Wilt.*—Tomato seedlings whose roots were allowed to remain in a thiolutin solution containing 10 to 80 p.p.m. of the antibiotic for four days and then inoculated with a spore suspension of the wilt organism (*Fusarium oxysporum* f. *lycopersici*) remained free of the disease (131). Plants placed in solutions containing 40 p.p.m. or more of the antibiotic showed distinct stunting.

## DISEASES OF TOBACCO

*Wildfire.*—Heggested & Clayton (132) reported that streptomycin sulfate gave excellent control of wildfire, caused by *Pseudomonas tabaci,* in tobacco plant beds.

The most efficient control resulted from Agri-mycin and Agristrep at the

rates of 200 p.p.m. In the plant beds treated with these antibiotics no plants were killed by wildfire, whereas seven per cent of the plants treated with bordeaux and 20 per cent of those treated with Tri-basic copper died from it. In the untreated checks, 30 per cent of the plants died. In the plots treated with Agristrep and Agri-mycin, leaf infection of the surviving plants was practically negligible; in those treated with the copper compounds, incidence of infection was considerable.

In addition to protecting tobacco plants from wildfire infection, streptomycin sulfate sprays eradicated the disease. Seriously infected plants in growers' beds were sprayed three times at weekly intervals with streptomycin sulfate at a concentration of 200 p.p.m. When the treatments were terminated, one-third more of the plants were surviving in the treated areas than in the untreated ones. Only five per cent of the leaves examined from untreated areas showed no wildfire infection, while 79 per cent of those from the sprayed plots were free of infection.

Beach & Engle (133) in 1955 reported excellent control of wildfire with Phytomycin in Pennsylvania. Tobacco seedlings were sprayed four times at intervals of 8 to 10 days with a solution containing 100 p.p.m. of streptomycin. Kirby (134), also in Pennsylvania, showed that Agri-mycin at a concentration of 100 p.p.m. of streptomycin plus ferbam, 3 lb. per 100 gal. of water, applied four times at weekly intervals prevented the development of wildfire in the seedbed and reduced the amount of infection in the field. When four weekly sprays at 200 p.p.m. were applied to infected plants, the spread of the wildfire organism was checked and thus the plants were enabled to grow normally.

Heggested, Neas & Grosso (135) found that treatments with streptomycin sulfate dust were not as effective as spray treatments in controlling the disease. Shaw & Thorne (136) in North Carolina, in 1955, reported that three or four applictions of a streptomycin spray containing 200 p.p.m. of the antibiotic at weekly intervals beginning immediately after inoculation with the wildfire organism reduced the incidence of the disease in the plant bed. A dust containing 1000 or 2000 p.p.m. of streptomycin applied in the same manner as the streptomycin spray was also effective in controlling the disease. In tests conducted in 1956 Shaw *et al.* (137) reported effective control with six weekly spray applications of the antibiotic at a concentration of 100 p.p.m. Dosages of 3 and 5 gal. spray per 100 sq. yd. gave equally effective control. Rhodes, Mullet & Mathews (138) indicated that four weekly applications of a 100-p.p.m. streptomycin spray were superior to the standard Tri-basic copper treatment for wildfire protection. Similar applications of a 0.2 per cent streptomycin dust also retarded infection. Natural field infection was eradicated with a spray containing 200 p.p.m. of the antibiotic.

*Blue mold.*—In greenhouse tests, Grosso (139) controlled blue mold, caused by *Peronospora tabacina,* with streptomycin sulfate. This was the first report of control of a fungus disease with this antibiotic. Young to-

bacco plants were sprayed with aqueous solutions of 10 antibiotics at 100 p.p.m. and with Dithane (Zineb). Four applications were made at three- or four-day intervals. The day after the second application, the plants were inoculated with spores of the blue-mold fungus. Streptomycin reduced leaf damage due to the blue-mold fungus to 1.8 per cent as compared with 30 per cent in the untreated check. Spraying with other antibiotics was not effective.

In tobacco beds, Anderson (140) reported good control of blue mold with sprays containing 200 or 400 p.p.m. of streptomycin applied once or twice a week during the development of the seedlings. After the spray program was stopped, blue mold appeared in less than a week. This suggested that streptomycin is fungistatic rather than fungicidal.

## DISEASES OF CEREALS

### Corn

*Bacterial wilt.*—Natti (141) reported that in field tests in New York one or two foliar spray applications of streptomycin at 100 p.p.m. did not reduce natural infection of Stewart's bacterial wilt of corn, caused by *Bacterium stewartii,* but that it prevented some secondary spread of the organism.

Williams & Lockwood (142) in greenhouse tests noted that streptomycin, oxytetracycline, and actidione afforded only slight control of the disease. Under field conditions, Lockwood & Williams (143) reduced the incidence and severity of the disease with sprays containing 100 p.p.m. of streptomycin or 50 p.p.m. of oxytetracycline. Six applications were made at approximately four-day intervals. Higher concentrations of the antibiotics gave greater disease control.

Rich (144) and Williams (145), soaking disease-free corn seed in water solutions of oxytetracycline, streptomycin, or tetracycline, immediately before sowing, found a reduction in the severity of the symptoms of the disease on seedlings inoculated at the two-leaf stage.

### Wheat

*Rust.*—Silverman & Hart (146) found in greenhouse tests that an actidione spray containing 20 to 25 p.p.m. of the antibiotic controlled wheat rust, caused by *Puccinia graminis* var. *tritici,* in seedlings by reducing the number of pustules and delaying sporulation. The antibiotic, however, caused a severe dieback of the seedling leaf tips. Wallen (147), using several antibiotics as sprays, found actidione the most promising for control of the disease. Concentrations of 50 to 500 p.p.m. were phytotoxic. The degree of infection ranged from 0 to 5 per cent in the plots sprayed every 10 days with a 500 p.p.m.-concentration, but from 50 to 60 per cent when the concentration of the spray was 25 p.p.m. All plants in the control plot were infected. The plots sprayed before heading, after heading, and at 10-day intervals consistently outyielded the unsprayed control plots.

Hacker & Vaughn (148, 149) reported that the semicarbazone of cycloheximide, a derivative of actidione, used as a spray at a concentration of 100 and 200 p.p.m. of the antibiotic, protected spring wheat from black-stem rust. The protection persisted for the life of the plant after treatment at the boot stage. The acetate of cycloheximide reacted similarly but did not impart complete protection.

*Powdery mildew.*—Powers (150) reported that anisomycin in an aqueous solution at 300 p.p.m. eradicated *Erysiphe graminis* from wheat seedlings. Applying the antibiotic as late as seven days after inoculation, when the mildew was well developed, eradicated infection. Anisomycin principally affected the external mycelium. Crosier & Szkolnik (151) noted that in greenhouse tests actidione as a foliar spray at a concentration of 2 or 3 p.p.m. reduced powdery mildew only slightly.

*Other diseases.*—Leben, Arny & Keitt (152), in small-scale greenhouse and field tests, found that seed treatment with crude helixin B controlled helminthosporium blights of oats (*Helminthosporium victoriae*) and of barley (*H. sativum*). In limited tests, the antibiotic was effective also in controlling wheat bunt (*Tilletia foetida*), oat smut (*Ustilago avenae*), and covered smut of barley (*Ustilago hordei*). Henry, Millar & Peterson (153) found that a rapid seed treatment of wheat with 0.5 and 1 per cent actidione dust mixed with Dixie clay at the rate of 0.5 oz. per bu. gave almost complete control of covered smut of wheat. Seedling emergence was not greater than that in the untreated check; in fact, when 1 per cent dust was used emergence was slightly reduced. Later, Henry *et al.* (154) demonstrated that when the organic fungicide captan, which alone did not give satisfactory control of the disease, was added to the mixture of actidione and Dixie clay, emergence was improved and the smut was well controlled.

Rangaswami (155) found that wheat seeds naturally infected with *Helminthosporium* sp. treated with mycothricin complex A at a concentration of 2500 p.p.m. completely inhibited the growth of the organism and increased seed germination.

Hagborg (156) observed that in culture solutions resistance of wheat seedlings to infection by *Xanthomonas translucens* was increased by streptomycin, but not by actidione, griseofulvin, or neomycin.

Sabet (157), spraying corn seedlings with streptomycin or oxytetracycline before inoculation with *Erwinia (Bacterium) carotovorum* f. *zeae*, reduced the incidence and severity of leaf blight. Oxytetracycline was more effective than streptomycin.

## DISEASES OF TURF GRASS

Vaughn & Klomparens (158) found that actidione gave excellent control of dollar spot of turf (*Sclerotinia homoeocarpa*) and of melting out of turf (*Helminthosporium vagus*). Howard (159) reported it to be effective in controlling the fading out disease of turf (*Curvularia lunata*). Couch & Cole (160) observed in 1956 that this antibiotic and oxytetracycline produced

highly significant reductions of the leaf-spot phase of the disease. They (161) also found that actidione gave satisfactory control of rust (*Puccinia graminis*) of Merion blue grass. The best control resulted with 422 or 844 mg. of the material dissolved in 2 gal. of water applied to 1000 sq. ft. of sod at 15-day intervals, but phytotoxicity was observed.

## DISEASES OF OIL SEED CROPS

*Bacterial leaf spot of sesame.*—Thomas (162) demonstrated in field tests in Maryland that soaking sesame seed infected with *Pseudomonas sesame* for 30 min. in an aqueous solution of Agri-mycin containing 250 p.p.m. of streptomycin completely controlled leaf spot. Plants grown from seeds soaked in water for the same period were almost completely defoliated by the leaf-spot organism.

*Damping-off of safflower.*—Gattani (163) showed that treating 7-day-old safflower seedlings in a solution containing 50 p.p.m. of filipin for 72 hr. before transplanting in soil infested with *Pythium* spp. considerably reduced seedling infection for four to six weeks. Seeds of safflower sown in *Pythium*-infested soil and subsequently treated with the antibiotic increased seedling survival for three to six weeks.

*Bacterial blight of soybean.*—In field tests, Kaufmann & Chamberlain (164) reported that soybeans sprayed with streptomycin at a concentration of 250 p.p.m. of the antibiotic gave satisfactory control of bacterial blight caused by *Pseudomonas glycinia.*

## DISEASES OF INDUSTRIAL CROPS

*Downy mildew of hop.*—Horner & Maier (165), spraying hops systemically infected with downy mildew (*Pseudoperonospora humuli*), eradicated the disease with streptomycin or griseofulvin at concentrations up to 2000 p.p.m. of the antibiotics.

*Mint rust.*—Nelson (166) demonstrated that under field conditions a 2 per cent actidione dust protected mint from rust (*Puccinia menthae*).

## DISEASES OF THE ORNAMENTAL PLANTS

*Bacterial wilt of chrysanthemum.*—Robinson, Starkey & Davidson (167) reported *Erwinia chrysanthemi,* the cause of bacterial wilt of chrysanthemum, to be controlled by streptomycin or oxytetracycline. Chrysanthemum cuttings were soaked in water solutions of the antibiotic for 6 hr., washed, inoculated with the bacterial wilt organism, and planted in sterilized sand. A high percentage of the treated cuttings survived, whereas all the untreated cuttings died after 25 days. Streptomycin was more effective than oxytetracycline.

Cuttings inoculated with the wilt organism and treated later with the two antibiotics showed a high survival after 20 days, whereas all the untreated cuttings were killed. As in the protection experiment, streptomycin gave better control than oxytetracycline.

In later studies, Robinson (168) reported that the application of a solution containing 10 p.p.m. of streptomycin to the rooting sand resulted in the survival and rooting of 50 per cent of chrysanthemum cuttings inoculated with the wilt organism; at 50 p.p.m. complete survival resulted. When the antibiotic was applied at 100 p.p.m. 93 per cent of initially infected cuttings survived.

*Leaf rot of philodendron.*—Miller (169) in studies on a bacterial leaf and stem rot of philodendron caused by *Erwinia* sp. found that a 200-p.p.m. Agri-mycin solution applied 48 hr. before infection completely prevented the development of symptoms. One aplication of a 400 p.p.m.-Agri-mycin solution after the symptoms were noted arrested further development.

*Snapdragon rust.*—Ark & Alcorn (53) reported candicidin fairly effective in controlling snapdragon rust (*Puccinia antirrhini*). When greenhouse-grown plants were sprayed with the antibiotic at a concentration of 200 p.p.m. immediately before inoculation, a very high degree of protection resulted.

## DISEASES OF FOREST AND SHADE TREES

*Oak wilt.*—Fergus, Cole & Stambaugh (170) noted that actidione applied as a spray at a concentration of 10 p.p.m. on oak sapwood infected with *Endoconidiophora fagacearum* reduced mycelial invasion by 90 per cent, suggesting its possible value in the prevention of mat formation.

Phelps, Kuntz & Riker (171) demonstrated that oligomycin, actidione, and its acetate derivative inserted into oak trees 24 hr. before wound inoculation with the oak wilt fungus delayed symptom expression in over half of the treated trees for at least three months. Ninety-five per cent of the controls showed symptoms in one month.

*Dutch elm disease.*—Holmes (172) was unable to prevent infection of the Dutch elm disease, caused by *Graphium ulmi,* in young elm trees with any of the 22 antibiotics tested. Among those used were candicidin A, patulin, pleocidin, polymyxin B, streptomycin, streptothricin, thiolutin, actidione, amphomycin, and chlortetracycline.

*White pine blister rust.*—As a damage-preventive measure in western white pine stands that may become infected by white pine blister rust (*Cronartium ribicola*) before *Ribes* are destroyed, Moss (173) found that actidione was effective in killing mycelium in the perimeter of excised trunk canker wounds. Actidione in oil was applied to the cut surface of wounds after the bark was removed to expose the mycelium.

## ROOT ROTS

Treating soils with actidione, fradicin, clavacin, and gliotoxin, Gregory *et al.* (174) found them to be active against *Pythium* spp. In alkaline and acid soils actidione was present for 8 and 14 days, respectively. Fradicin remained in both acid and alkali soils after 14 days. Gliotoxin was not detected after a few hours, while clavacin remained one day in alkaline soils

and two to four days in the acid. Verona & Gambogi (175) reported that nystatin gave satisfactory control of a natural infection of clover seedlings by *Pythium* spp.

## VIRUS DISEASES OF PLANTS

Thus far, only limited investigations have been conducted on the inhibition of plant viruses by antibiotics. Leben & Fulton (176), in detached-leaf studies, demonstrated that streptomycin and oxytetracycline both prevented the production of local lesions on cowpea leaves by tobacco necrosis and tobacco ring-spot viruses. Only partial inhibition of the TMV was obtained. The writers suggested that the inhibitory effects were due to action on the host, since the antibiotics did not influence the infectivity of the viruses tested *in vitro*.

Schegel & Rawlins (177) studied the effect of MK61 on leaf discs infected with TMV and floated on solutions of this antibiotic. Quantitative data showed that the antibiotic at 10 p.p.m. and higher concentrations caused 69 to 90 per cent inhibition of virus infectivity. The investigators assumed that it directly affected virus multiplication rather than host metabolism.

Gray (178) reported that spraying leaves with a solution of purified noformicin at a concentration of 125 p.p.m. delayed and inhibited markedly both local-lesion production and systemic infection by southern bean mosaic virus in beans and TMV in *Nicotiana rustica* and tobacco. The antibiotic did not, however, destroy the viruses as multiplication resumed after applications of the antibiotic were stopped. Application of the antibiotic to roots of Pinto bean plants growing in sand reduced the number of local lesions produced on the inoculated primary leaves.

Local-lesion production by southern bean mosaic virus and TMV was completely prevented by cytovirin sprays of 0.5 to 1 p.p.m. concentration. Protection from systemic infections by the tomato spotted wilt virus and TMV resulted from spraying the plants twice with 100 p.p.m. of the crude antiviral agent (179, 180).

## MODE OF ACTION

Anderson & Nienow (181) in 1947 demonstrated that streptomycin was absorbed by soybean plants growing in a nutrient solution containing the antibiotic. The extracted sap contained 4 to 5 p.p.m. of the antibiotic. Brian *et al.* (124) found that griseofulvin was absorbed by lettuce seedlings from nutrient solutions containing the antibiotic and that these plants were less susceptible to infection by *Botrytis cinerea* than the control plants. Tomatoes grown in sand cultures to which a 500 p.p.m.-solution of griseofulvin had been added developed only barely visible pin-point lesions when inoculated with the early blight fungus (*Alternaria solani*). The period of complete protection given by this treatment was less than 14 days. Mitchell, Zaumeyer & Preston (182, 183), having applied streptomycin in a lanolin paste to the stems of bean plants, detected the antibiotic in the juice of nearby leaves.

When filter-paper discs impregnated with this juice were placed on agar plates seeded with *Xanthomonas phaseoli*, inhibition zones became evident around the discs. Similarly, a streptomycin-dependent strain of *Escherichia coli* grew on agar that contained the diffusate from the juice of plants whose stems had been treated with streptomycin sulfate but did not grow on agar that contained the diffusate from the juice of untreated plants.

Streptomycin was rapidly absorbed by the stems and translocated to primary leaves, for measurable amounts of the free antibiotic were detected within 8 hr. after treatment. Concentration of the free antibiotic was greatest during the third day. It was shown that as much as 67 per cent of the total streptomycin applied to the stems was translocated to the primary leaves as free streptomycin during the five days immediately following treatment.

Napier *et al.* (184) in England also found that streptomycin exerted a marked systemic action against the halo blight organism even at sites as remote from the point of application as the fourth trifoliate leaf. The systemic protection persisted up to 11 days.

Robinson *et al.* (167) showed that both streptomycin and oxytetracycline were readily absorbed and translocated in chrysanthemum cuttings and less readily in rooted plants. In treated cuttings the antibiotics persisted in sufficient amounts to protect the newly developing plants from infection by *Erwinia chrysanthemi*. Streptomycin remained active in plants for several weeks.

Pramer (185) and Crowdy & Pramer (186) found that some antibiotics were readily aborbed by some plant species but that others were not. Distribution within the plants varied with both plant species and antibiotic. Some of the antibiotics translocated moved more readily than others in all plants tested. Some appeared to move more freely in certain plants than in others. Readily translocated antibiotics are either neutral or acidic substances (griseofulvin and penicillin).

Goodman & Hemphill (187) reported that when indole-3-acetic acid (IAA) was added to an antibiotic spray formulation, containing 250 p.p.m. each of streptomycin and oxytetracycline and 1 per cent each of methyl cellosolve and Carbowax 4000 fire blight control was markedly increased in apple shoots inoculated 24 hr. after application of the spray. Later Hemphill & Goodman (188) demonstrated that the ethyl ester was just as effective as indole-3-acetic acid, in concentrations as low as 50 p.p.m. They suggested that the property of increasing the efficacy of antibiotics is not restricted to these two compounds but is shared by a large group of plant growth regulators. Most of these materials demonstrated activity similar to that of indole-3-acetic acid and its ethyl ester. Hemphill & Goodman concluded that the increased disease control was not effected by action of the growth-regulating substances on the pathogen but reflects some host-plant reaction to the growth-regulating substance such as increased water uptake or increased

permeability of cell membranes, each of which might facilitate the movement of the antibiotic. Also, an increase in metabolic rate resulting from application of these substances may speed maturation and reduce susceptibility of the tissues.

Crowdy, Grove & Pramer (189), in studies on the systemic distribution of antibiotics in *Vicia faba* plants, recovered pure griseofulvin from the tops of plants whose roots were treated with the antibiotic. They showed that the uptake of griseofulvin and chloramphenicol is linearly related to the uptake of water in the plant. Griseofulvin showed no tendency to accumulate in the roots or stems, but there was a marked accumulation in the leaves. The distribution of chloramphenicol within the plants was similar to that of griseofulvin. No streptomycin could be detected in the top of plants grown for 67 hr. in solutions containing 200 p.p.m. of the antibiotic and only a trace in those grown for 137 hr. All the streptomycin absorbed by broad beans was concentrated in the lower leaves. Top leaves of tomato plants grown in streptomycin solutions contained the antibiotic, but greater concentrations were found in the lower leaves.

Muller, Mackay & Friend (190) demonstrated that infection by *Phytophthora infestans* was completely checked in tomato plants inoculated with the organism after they had remained in a culture solution containing 4 p.p.m. of streptomycin for one week. Infection was not prevented, but spread of the pathogen was restricted after contact with the host plasma. The concentration of streptomycin supplied and those in extracts of treated plants showed no significant effect on zoospore development or their germination, and the mycelium tolerated the antibiotic at concentrations of 200 p.p.m. Muller *et al.* assumed that streptomycin induces some change which alters the host-pathogen relationship in a way such as to confer some degree of resistance on the host.

Gray (191) demonstrated that streptothricin and pleocidin, which were readily absorbed by leaves of tobacco, tomato, and bean plants when sprayed on the intermediate leaves, were translocated out of the leaves and not only up the stem to the young leaves, but also down to older untreated leaves and in some cases to the roots. Under the same conditions no translocation of streptomycin, neomycin, bacitracin, or actinomycin was detected. Where one primary leaf of a bean plant was immersed in a vial containing 2000 p.p.m. of the antibiotic in an aqueous solution, movement of chloramphenicol, penicillin, streptomycin, streptothricin, pleocidin, and viomycin out of the treated leaf and up to the young trifoliate leaves occurred. Movement of all these except chloramphenicol across the stem to the opposite primary leaf also occurred.

Using a spray containing 500 p.p.m. of streptomycin and 1 per cent glycerol, Gray (192, 193) found that much greater absorption of the antibiotic occurred in 3 hr. after application to bean leaves than if streptomycin was used alone. After 6 hr. the absorption was five times as great and after

96 hr. 24 times as great. The absorption of chloramphenicol and streptothricin also was increased by the addition of glycerol, but not that of oxytetracycline and neomycin. Gray also showed that a number of polyhydroxyl alcohols which exhibit low volatility properties increased absorption.

Alcorn & Ark (194, 195) reported more rapid translocation of chlortetracycline, oxytetracycline, and tetracycline than of streptomycin and neomycin in *Pyracantha* cuttings. In stems of carnation cuttings also, tetracycline moved more rapidly than streptomycin. Adding 1 per cent $K_2HPO_4$ to streptomycin solutions in which carnation cuttings were placed increased the rapidity and the distance of movement of the antibiotic.

Pramer, Robinson & Starkey (196) showed that the control of the bacterial wilt of chrysanthemum by streptomycin resulted from the direct action of the antibiotic on the pathogen. Recent reports by Bonde (111) and Muller *et al.* (190) describing the control of *Phytophthora infestans* on potato and tomato *in vivo* by streptomycin, which has no activity on these pathogens *in vitro,* can be explained only by an indirect mode of action of the antibiotic.

Dye (197) placed roots of peach seedlings in nutrient solutions containing 10, 25, and 50 p.p.m. of streptomycin and observed that the antibiotic passed into the foliage where it sometimes reached concentrations higher than those of the solutions surrounding the roots. In all plants the concentrations of the antibiotic present in the upper leaves were less than in the lower leaves.

Dye (198) studying leaf absorption of streptomycin in peach leaves, noted that the antibiotic enters the leaves following foliar applications, but he found no evidence that it becomes truly systemic.

Hidaka & Murano (199, 200) found that the lower surfaces of tobacco leaves treated with streptomycin solutions absorbed twice as much antibiotic as the similarly treated upper surfaces. The rate of absorption was correlated with the concentration of the antibiotic solution. When applied to the stem of tobacco plants, the antibiotic moved into the phloem and pith and was translocated upward in these tissues. It could not be traced in xylem vessels.

Zaumeyer (201) in studies on the control of bean rust reported that when the lower surfaces of bean leaves were treated with oligomycin or anisomycin they were better protected against infection by *Uromyces phaseoli typica* than when the upper surfaces were treated. Excellent protection against infection by *Colletotrichum lindemuthianum* resulted from treating lower surfaces, but treatment of upper surfaces gave no protection. Better absorption of these antibiotics by the lower surfaces than by the upper accounted for this increased protection.

Wallen & Millar (202) demonstrated that cycloheximide applied as a spray to leaves of wheat seedlings was absorbed and could be recovered

from them for at least five weeks after application. When supplied to seedling roots in sand or soil, the antibiotic was absorbed by them and translocated throughout the stems and leaves.

Maier & Horner (203) in studies on hops noted that streptomycin was readily absorbed and translocated when applied to the stems. The antibiotic attained a mean concentration of 46 p.p.m. after 24 hr. and declined to 25 p.p.m. after 72 hr., but persisted at a concentration of 1.2 p.p.m. for 12 days.

Goodman & Dowler (204) found that naphthyl acetamide and gibberellic acid, as well as glycerol, diethylene glycol, or triethylene glycol, when added to streptomycin increased the absorption of the antibiotic by bean plants. Indole-3-acetic acid and a combination of methyl cellosolve and Carbowax 4000, when formulated with streptomycin, reduced the absorption of the antibiotic. The exact nature of the factors responsible for increasing the absorption of streptomycin by these materials is unknown.

Aytoun (205) studied the effects of griseofulvin on the development of *Botrytis tulipae* in tulip when the antibiotic was taken into the plant by root absorption. He demonstrated that the mycelium of the pathogen developed morphologic abnormalities within the tissues which contained griseofulvin and that it was unable to pass from cell to cell through plant membranes.

Natti (90) in his studies on the control of downy mildew of broccoli considered the action of streptomycin as a typical systemic type. Control of the fungus by the antibiotic was attributed to direct toxicity of the antibiotic to the fungus or to changes in the metabolism of the plant induced by streptomycin to render the plants resistant. Streptomycin appeared to enter the plants by diffusion. Any condition that maintains streptomycin in a soluble form on the foliage favors its entrance into the plant. Transpiration did not appear to be directly involved in the movement of the antibiotic into the plant. More streptomcyin entered broccoli plants in a saturated atmosphere when transpiration is usually neglible than under conditions of low relative humidity which favors high rates of transpiration.

## LITERATURE CITED

1. Weindling, R., *Phytopathology*, **22**, 837–45 (1932)
2. Weindling, R., *Phytopathology*, **24**, 1153–79 (1934)
3. Weindling, R., and Emerson, O. H., *Phytopathology*, **26**, 1068–70 (1936)
4. Brown, J. G., and Boyle, A. M., *Science*, **100**, 528 (1944)
5. Brown, J. G., and Boyle, A. M., *Phytopathology*, **34**, 760–61 (1944)
6. Brian, P. W., and Hemming, H. G., *Ann. Appl. Biol.*, **32**, 214–20 (1945)
7. Brown, J. G., and Heep, D. M., *Science*, **104**, 208 (1946)
8. Zaumeyer, W. J., Thomas, H. R., Mitchell, J. W., and Fisher, H. H., *Phytopathology*, **43**, 407 (1953)
9. Ark, P. A., *Plant Disease Reptr.*, **37**, 404–6 (1953)
10. Weindling, R., Katznelson, H., and Beale, H. P., *Ann. Rev. Microbiol.*, **4**, 247–60 (1950)
11. Anderson, H. W., and Gottlieb, D., *Econ. Botany*, **6**, 294–308 (1952)
12. Thomas, H. R., *Soil Sci. Soc. Florida, Proc.*, **13**, 17–25 (1953)

13. Leben, C., and Keitt, G. W., *J. Agr. Food Chem.*, **2**, 234–39 (1954)
14. Zaumeyer, W. J., *J. Agr. Food Chem.*, **3**, 112–16 (1955)
15. Zaumeyer, W. J., *Proc. Intern. Conf. Antibiotics Agr., 1st Conf.*, **1**, 171–87 (1955)
16. Murneek, A. E., *Phytopathology*, **42**, 57 (1952)
17. Goodman, R. N., *Am. Fruit Grower*, **73**, 7, 16, 17 (1953)
18. Goodman, R. N., *Proc. Am. Soc. Hort. Sci.*, **64**, 186–90 (1954)
19. Goodman, R. N., *Plant Disease Reptr.*, **38**, 874–78 (1954)
20. Young, H. C., and Winter, H. F., *Plant Disease Reptr.*, **37**, 463–64 (1953)
21. Heuberger, J. W., and Poulos, P. L., *Plant Disease Reptr.*, **37**, 81–83 (1953)
22. Ark, P. A., *Ind. Microbiol. Soc., Proc., 12th Gen. Meet.*, 34 (1955)
23. Ark, P. A., and Scott, C. E., *Phytopathology*, **44**, 481 (1954)
24. Dunegan, J. C., Kienholz, J. R., Wilson, R. A., and Morris, W. T., *Plant Disease Reptr.*, **38**, 666–69 (1954)
25. Kienholz, J. R., *Plant Disease Reptr.*, **39**, 208–9 (1955)
26. Clayton, C. N., *Plant Disease Reptr.*, **39**, 128–31 (1955)
27. Kirby, R. S., *Plant Disease Reptr.*, **38**, 432–33 (1954)
28. Mills, W. D., *Plant Disease Reptr.*, **39**, 206–7 (1955)
29. Miller, P. M., *Plant Disease Reptr.*, **41**, 19–22 (1957)
30. Goodman, R. N., *Plant Disease Reptr.*, **39**, 922–25 (1955)
31. Ark, P. A., *Plant Disease Reptr.*, **39**, 926–28 (1955)
32. Hamilton, J. M., and Szkolnik, M., *Phytopathology*, **43**, 109 (1953)
33. Cation, D., *Phytopathology*, **43**, 468 (1953)
34. McClure, T. T., *Phytopathology*, **42**, 14 (1952)
35. McClure, T. T., and Cation, D., *Plant Disease Reptr.*, **35**, 393 (1951)
36. Petersen, D., and Cation, D., *Plant Disease Reptr.*, **34**, 5–6 (1950)
37. Gilmer, R. M., *Plant Disease Reptr.*, **39**, 762–70 (1955)
38. Prescott, G. C., Emerson, H., and Ford, J. H., *Ind. Microbiol. Soc., Proc., 12th Gen. Meet.*, 42 (1955)
39. Hamilton, J. M., Szkolnik, M., and Sondheimer, E., *Science*, **123**, 1175–76 (1956)
40. Crosse, J. E., and Bennett, M., *Plant Pathol.*, **6**, 31–35 (1957)
41. Dye, E. W., and Dye, M. H., *New Zealand J. Sci. Technol.*, **A36**, 21–26 (1954)
42. Dye, M. H., *Orchardist New Zealand*, **29**, 2–3 (1956)
43. Goodman, R. N., and Shepard, P., *Plant Disease Reptr.*, **40**, 93–102 (1956)
44. Daines, R. H., *Plant Disease Reptr.*, **40**, 335–36 (1956)
45. Daines, R. H., and Gray, R., *Phytopathology*, **47**, 448 (1957)
46. Dunegan, J. C., and Wilson, R. A., *Phytopathology*, **43**, 405 (1953)
47. Dunegan, J. C., Wilson, R. A., and Morris, W. T., *Plant Disease Reptr.*, **37**, 604–5 (1954)
48. Alcorn, S. M., and Ark, P. A., *Plant Disease Reptr.*, **39**, 210–12 (1955)
49. Szkolnik, M., and Hamilton, J. M., *Plant Disease Reptr.*, **41**, 289–92 (1957)
50. Di Marco, G. R., and Davis, B. H., *Plant Disease Reptr.*, **41**, 284–92 (1957)
51. Ogawa, J. M., and Vergara, C., *Phytopathology*, **45**, 695 (1955)
52. Strong, F. C., and Klomparens, W., *Plant Disease Reptr.*, **39**, 569 (1955)
53. Ark, P. A., and Alcorn, S. M., *Plant Disease Reptr.*, **40**, 85–92 (1956)
54. Mirzabekian, R. O., *Agrobiologiya* **2**, 130–34 (1955)
55. Brown, J. G., and Boyle, A. M., *Phytopathology*, **35**, 521–24 (1945)
56. Brown, J. G., *Phytopathology*, **38**, 3 (1948)

57. Hampton, J. E., *Phytopathology*, **38**, 11 (1948)
58. Blanchard, F. A., *Phytopathology*, **41**, 955–58 (1951)
59. Klemmer, H. W., Riker, A. J., and Allen, O. N., *Phytopathology*, **45**, 618–25 (1955)
60. Deep, I. W., *Phytopathology*, **46**, 635 (1956)
61. Zuckerman, B., *Plant Disease Reptr.*, **41**, 674–77 (1957)
62. Ark, P. A., and Sibray, W. S., *Plant Disease Reptr.*, **41**, 449–51 (1957)
63. Ark, P. ., *Diamond Walnut News*, **35**, 6 (1953)
64. Ark, P. A., and Charles, F. M., *Calif. Agr.*, **10**, 8 (1956)
65. Smith, W. J., *J. Colo. Wyo. Acad. Sci.*, **4**, 49 (1949)
66. Hildreth, R. C., and Starr, G. H., *J. Colo. Wyo. Acad. Sci.*, **4**, 58 (1950)
67. Starr, G. H., Burke, D., Smith, W., Hildreth, R., and Paulus, A., *Agron. J.*, **43**, 617 (1951)
68. Klinkowski, M., Kohler, H., and Schrodter, H., *Phytopathol. Z.*, **23**, 345–80 (1955)
69. Mitchell, J. W., Zaumeyer, W. J., and Anderson, W. P., *Science*, **115**, 114-15 (1952)
70. Zaumeyer, W. J., Thomas, H. R., and Mitchell, J. W., *Am. Vegetable Grower*, **1**, 5 (1953)
71. Dye, D. W., *New Zealand J. Sci. Technol.*, **A,37** 458–61 (1956)
72. Zaumeyer, W. J., *J. Agr. Food Chem.*, **3**, 112–16 (1955)
73. Woolliams, G. E., *Phytopathology*, **47**, 538 (1957)
74. Marlatt, R. B., *Plant Disease Reptr.*, **38**, 705–9 (1954)
75. Andersen, A. L., *Phytopathology*, **47**, 515 (1957)
76. Gray, R. A., *Plant Disease Reptr.*, **39**, 567–68 (1955)
77. Silber, G., and Kainski, J., *Phytopathology*, **46**, 242 (1956)
78. Felber, I. M., and Hamner, C. L., *Botan. Gaz.*, **110**, 324–25 (1948)
79. Zaumeyer, W. J., *Antibiotics Ann.*, 1015–18 (1956–57)
80. Alcorn, S. M., and Ark, P. A., *Plant Disease Reptr.*, **38**, 705–9 (1954)
81. Zaumeyer, W. J., and Wester, R. E., *Phytopathology*, **46**, 470 (1956)
82. Ziffer, J., Ishihara, S. J., Cairney, T. J., and Chow, A. W., *Phytopathology*, **47**, 539 (1957)
83. Grummer, G., and Mach, F., *Zentr. Bakteriol. Parasitenk., Abt. 2*, 108, 449–64 (1955)
84. Zaumeyer, W. J., and Wester, R. E., *Phytopathology*, **46**, 32 (1956)
85. Zaumeyer, W. J., and Wester, R. E., *Plant Disease Reptr.*, **40**, 776–80 (1956)
86. Crossan, D. F., Lloyd, P. J., Hyre, R. A., and Heuberger, J. W., *Plant Disease Reptr.*, **41**, 156–59 (1957)
87. Cox, R. S., *Plant Disease Reptr.*, **39**, 484–86 (1955)
88. Natti, J. J., Hervey, G. E. R., and Sayre, C. B., *Plant Disease Reptr.*, **40**, 118–24 (1956)
89. Natti, J. J., and Hervey, G. E. R., *Phytopathology*, **46**, 242 (1956)
90. Natti, J. J., *Plant Disease Reptr.*, **41**, 780–88 (1957)
91. Sutton, M. D., and Bell, W., *Plant Disease Reptr.*, **38**, 547–52 (1954)
92. Ark, P. A., *Phytopathology*, **37**, 842 (1947)
93. Doolittle, S. P., and Beecher, F. S., *Plant Disease Reptr.*, **39**, 731–36 (1955)
94. Williams, L. E., and Lockwood, J. L., *Plant Disease Reptr.*, **40**, 479–82 (1956)
95. Ark, P. A., and Thompson, J. B., *Plant Disease Reptr.*, **41**, 452–54 (1957)
96. Coe, D. M., *Plant Disease Reptr.*, **39**, 729–30 (1955)

97. de Zeeuw, D. J., and Vaughn, J. R., *Plant Disease Reptr.*, **34,** 7–8 (1950)
98. Nelson, R., *Phytopathology,* **41,** 28 (1951)
99. Dekker, J., *Nature,* **175,** 689–90 (1955)
100. Bonde, R., *Am. Potato J.,* **30,** 143–47 (1953)
101. Bonde, R., and de Souza, P., *Am. Potato J.,* **31,** 311–16 (1954)
102. Bonde, R., *Plant Disease Reptr.,* **39,** 120–23 (1955)
103. Epps, W. M., *Plant Disease Reptr.,* **41,** 148–50 (1957)
104. Robinson, D. B., and Hurst, R. R., *Am. Potato J.,* **33,** 56–59 (1956)
105. Bonde, R., and Malcolmson, J. F., *Plant Disease Reptr.,* **40,** 615–19 (1956)
106. Malcolmson, J. F., and Bonde, R., *Plant Disease Reptr.,* **40,** 708–13 (1956)
107. Van Schaack, V., *Phytopathology,* **38,** 27 (1948)
108. Perkins, B. L., *J. Colo.-Wyo. Acad. Sci.,* **4,** 57 (1950)
109. Bonde, R., *Maine Farm Research,* **1,** 18–20 (1953)
110. Logsdon, C. E., *Phytopathology,* **47,** 22 (1957)
111. Bonde, R., *Phytopathology,* **43,** 463–64 (1953)
112. Hodgson, W. A., *Am. Potato J.,* **33,** 185-86 (1956)
113. Smith, W. L., Jr., *Phytopathology,* **42,** 475 (1952)
114. Smith, W. L., Jr., *Phytopathology,* **45,** 88-90 (1955)
115. Conover, R. A., *Plant Disease Reptr.,* **38,** 405–9 (1954)
116. Coe, D. M., *Plant Disease Reptr.,* **39,** 215-18 (1955)
117. Conover, R. A., *Plant Disease Reptr.,* **39,** 611–15 (1955)
118. Cox, R. S., Comegys, W. A., and Heuberger, J. W., *Bull. State Board Agri. (Delaware),* **43,** (1953)
119. Crossan, D. F., and Krupka, L. R., *Plant Disease Reptr.,* **39,** 480–89 (1955)
120. Cox, R. S., *Plant Disease Reptr.,* **39,** 616 (1955)
121. Cox, R. S., *Plant Disease Reptr.,* **40,** 205–9 (1956)
122. Cox, R. S., *Phytopathology,* **47,** 6 (1957)
123. Altman, J., and Davis, B. H., *Phytopathology,* **45,** 183 (1955)
124. Brian, P. W., Wright, J. M., Stubbs, J., and Wray, A. M., *Nature,* **167,** 347 (1951)
125. Stubbs, J., *Ann. Appl. Biol.,* **39,** 439 (1952)
126. Davis, D., and Rothrock, J. W., *Plant Disease Reptr.,* **40,** 328–31 (1956)
127. Gottlieb, D., Bhattacharyya, P. K., Carter, H. E., and Anderson, H. W., *Phytopathology,* **41,** 393 (1951)
128. Stessel, G. J., Leben, C., and Keitt, G. W., *Phytopathology,* **43,** 23–26 (1953)
129. Lockwood, J. L., Leben, C., and Keitt, G. W., *Phytopathology,* **44,** 438–46 (1954)
130. Zaumeyer, W. J., and Doolittle, S. P., *Phytopathology,* **46,** 32 (1956)
131. Gopalkrishnan, K. S., and Jump, J. A., *Phytopathology,* **42,** 338–40 (1952)
132. Heggested, H. E., and Clayton, E. E., *Plant Disease Reptr.,* **38,** 661–65 (1954)
133. Beach, W. S., and Engle, H. B., *Plant Disease Reptr.,* **39,** 15–16 (1955)
134. Kirby, W. A., *Plant Disease Reptr.,* **39,** 14 (1955)
135. Heggested, H. E., Neas, M. O., and Grosso, J., *Plant Disease Reptr.,* **40,** 48–51 (1956)
136. Shaw, L., and Thorne, G. W., Jr., *Plant Disease Reptr.,* **45,** 325–27 (1956)
137. Shaw, L., Lucas, G. B., and Thorne, G. F., Jr., *Plant Disease Reptr.,* **41,** 99–102 (1957)
138. Rhodes, G. N., Mullet, R. P., and Mathews, J. N., *Plant Disease Reptr.,* **40,** 202–4 (1956)

139. Grosso, J. J., *Plant Disease Reptr.*, **38**, 333 (1954)
140. Anderson, P. J., *Phytopathology*, **46**, 240 (1956)
141. Natti, J. J., *Plant Disease Reptr.*, **39**, 386–90 (1955)
142. Williams, L. E., and Lockwood, J. L., *Phytopathology*, **47**, 44–47 (1957)
143. Lockwood, J. L., and Williams, L. E., *Plant Disease Reptr.*, **40**, 622–24 (1956)
144. Rich, S., *Plant Disease Reptr.*, **40**, 417–20 (1956)
145. Williams, L. E., *Plant Disease Reptr.*, **41**, 919–22 (1947)
146. Silverman, W. B., and Hart, H., *Phytopathology*, **44**, 506 (1954)
147. Wallen, V. R., *Plant Disease Reptr.*, **39**, 124–27 (1955)
148. Hacker, R. G., and Vaughn, J. R., *Plant Disease Reptr.*, **41**, 442–46 (1957)
149. Hacker, R. G., and Vaughn, J. R., *Phytopathology*, **47**, 14 (1957)
150. Powers, H. R., Jr., *Phytopathology*, **47**, 453 (1957)
151. Crosier, W., and Szkolnik, M., *Plant Disease Reptr.*, **40**, 337–39 (1956)
152. Leben, C., Arny, D. C., and Keitt, G. W., *Phytopathology*, **43**, 391–94 (1953)
153. Henry, A. W., Millar, R. L., and Peterson, E. A., *Science*, **115**, 90–91 (1952)
154. Henry, A. W., Peterson, E. A., and Millar, R. L., *Phytopathology*, **43**, 461 (1953)
155. Rangaswami, G., *Plant Disease Reptr.*, **40**, 483–87 (1956)
156. Hagborg, W. A. F., *Can. J. Microbiol.*, **2**, 80–86 (1956)
157. Sabet, K. A., *Ann. Appl. Biol.*, **44**, 152–60 (1956)
158. Vaughn, J. R., and Klomparens, W., *Phytopathology*, **42**, 22 (1952)
159. Howard, F. L., *Phytopathology*, **43**, 109 (1953)
160. Couch, H. B., and Cole, H., Jr., *Phytopathology*, **47**, 6 (1957)
161. Couch, H. B., and Cole, H., Jr., *Plant Disease Reptr.*, **40**, 103–5 (1956)
162. Thomas, C. H., *Phytopathology*, **46**, 29 (1956)
163. Gattani, M. L., *Plant Disease Reptr.*, **41**, 160–64 (1957)
164. Kaufmann, M. J., and Chamberlain, D. W., *Plant Disease Reptr.*, **41**, 806–7 (1957)
165. Horner, C. E., and Maier, C. R., *Phytopathology*, **47**, 525 (1957)
166. Nelson, R., *Phytopathology*, **41**, 27–28 (1951)
167. Robinson, R. S., Starkey, R. L., and Davidson, O. W., *Phytopathology*, **44**, 646–50 (1954)
168. Robinson, R. S., *Dissertation Abstr.*, **15**, 1701 (1955)
169. Miller, H. N., *Proc. Florida State Hort. Soc.*, **68**, 354–58 (1955)
170. Fergus, C. L., Cole, H., Jr., and Stambaugh, W. J., *Plant Disease Reptr.*, **39**, 491–94 (1955)
171. Phelps, W. R., Kuntz, J. E., and Riker, A. J., *Phytopathology*, **47**, 27 (1957)
172. Holmes, F. W., *Phytopathology*, **45**, 185 (1955)
173. Moss, V. D., *Plant Disease Reptr.*, **41**, 709–14 (1957)
174. Gregory, K. F., Allen, O. N., Riker, A. J., and Peterson, W. H., *Am. J. Botany*, **39**, 405–15 (1952)
175. Verona, O., and Gambogi, P., *Ann. Sperimentazione Agraria* (*Rome*), **11**, 193–209 (1957)
176. Leben, C., and Fulton, M. W., *Phytopathology*, **42**, 331–37 (1952)
177. Schlegel, D. E., and Rawlins, T. E., *Phytopathology*, **44**, 328–29 (1954)
178. Gray, R. A., *Phytopathology*, **45**, 281–85 (1955)
179. Gray, R. A., *Phytopathology*, **47**, 522 (1957)
180. Gray, R. A., *Plant Disease Reptr.*, **41**, 576–78 (1957)
181. Anderson, H. W., and Nienow, I., *Phytopathology*, **37**, 1 (1947)

182. Mitchell, J. W., Zaumeyer, W. J., and Preston, W. H., *Phytopathology,* **43,** 480 (1953)
183. Mitchell, J. W., Zaumeyer, W. J., and Preston, W. H., *Phytopathology,* **44,** 25-30 (1954)
184. Napier, E. J., Turner, D. I., Rhodes, A., and Toothhell, J. P. R., *Ann. Appl. Biol.,* **44,** 145–51 (1956)
185. Pramer, D., *Ann. Appl. Biol.,* **40,** 617–22 (1953)
186. Crowdy, S. H., and Pramer, D., *Chem. & Ind. (London),* **7,** 160–62 (1955)
187. Goodman, R. N., and Hemphill, D. D., *Science,* **119,** 347–48 (1954)
188. Hemphill, D. D., and Goodman, R. N., *Science,* **122,** 122 (1955)
189. Crowdy, S. H., Grove, J. F., and Pramer, D., *Intern. Botan. Congr., 8th Congr., Paris, Repts. Communications,* 73–82 (1954)
190. Muller, K. O., Mackay, J. H. E., and Friend, J. E., *Nature,* **174,** 878–79 (1954)
191. Gray, R. A., *Plant Physiology,* 30 (1955)
192. Gray, R. A., *Ind. Microbiol. Soc. Proc.,* 41 (1955)
193. Gray, R. A., *Phytopathology,* **46,** 105–11 (1956)
194. Alcorn, S. M., and Ark, P. A., *Ind. Microbiol. Soc., Proc., 12th Gen. Meet.,* 35 (1955)
195. Alcorn, S. M., and Ark, P. A., *Appl. Microbiol.,* **4,** 126–30 (1956)
196. Pramer, D., Robinson, R. S., and Starkey, R. L., *Phytopathology,* **46,** 341–42 (1956)
197. Dye, M. H., *Ann. Appl. Biol.,* **44,** 567–75 (1956)
198. Dye, M. H., *New Zealand Sci. Rev.,* **14,** 122–23 (1956)
199. Hidaka, Z., and Murano, H., *Phytopathol. Soc. Japan Ann.,* **20,** 143–47 (1956)
200. Hidaka, Z., and Murano, H., *Phytopathol. Soc. Japan Ann.,* **21,** 49–54 (1956)
201. Zaumeyer, W. J., *Phytopathology,* **47,** 539 (1957)
202. Wallen, V. R., and Millar, R. L., *Plant Disease Reptr.,* **41,** 291–94 (1957)
203. Maier, C. R., and Horner, C. E., *Phytopathology,* **47,** 528 (1957)
204. Goodman, R. N., and Dowler, A. M., *The Influence of Plant Growth Regulating Substances and Hydroxy-Compounds on the Absorption of Streptomycin by Plants* (Presented at 4th Intern. Congr. of Crop. Protection, Hamburg, Germany, 1957; in press)
205. Aytoun, R. S. C., *Ann. Botany (London),* (In press)

# MASKED VIRUS INFECTION IN PLANTS[1]

By E. M. Hildebrand

*Plant Pathologist, Crops Research Division, Agricultural Research Service, U. S. Department of Agriculture, Beltsville, Maryland*

## INTRODUCTION

By definition, masking is the presence of a living ultramicroscopic virus entity within the cytoplasm of living cells of a multicellular host plant, in the absence of distinctive symptoms. In the growth and development of the science of plant virology, the literature on plant virus diseases is replete with references to masking or other failure to produce distinctive symptoms. Possibly the practical considerations sometimes overshadow the scientific for recognizing and differentiating between plant virus diseases. Being completely intracellular and obligate parasites, we usually recognize virus diseases largely through symptom expression.

What was true in the early days of plant virology, holds even today; namely, the possibility of other causal agents must be removed from the scene and transmissibility has to be demonstrated before one can, with any assurance, label a disorder a virus disease. From this it follows that a prime essential for virus study is the propagation of healthy plants. Masked virus infections frequently complicated the problem of providing disease-free plants for virological research and, in turn, help focus attention on the masking phenomenon.

In a review such as this, one cannot hope to deal with all of the virus disease literature because the field is too extensive and too inclusive. Many viruses which have been transmitted experimentally to a rather wide range of host plants have infected, or multiplied in, some species in which no visible symptoms are produced, but from which virus can be recovered. Manifestly, there are many gaps in the literature where the lack of information on masking results from imperfect or inefficient experimental methods. Nevertheless, whether the difficulties of method be imaginary or real, virus masking can play a significant role for some viruses in nearly every crop plant.

In this review, by considering selected diseases, an attempt has been made to portray the role of masking in the syndrome picture as conditioned by host-growth status, seasonal development, and environment.

A successful virus must, a priori, establish and maintain itself in an harmonious relationship with the host plant. This harmony concerns (*a*) the relation of the virus component to the cell components; and (*b*) the tolerance range of the cell components to the virus invader, and vice versa. Per-

[1] The survey of the literature pertaining to this review was concluded in December, 1957.

fect harmony would constitute latency. By definition, latency is the presence of virus in the host with the total absence of visible symptoms over the entire range of environment to which the host or carrier is exposed. Thus, virus latency is a matter of duration, masking a partial latency. Theoretically, a long-latency virus should be practically indistinguishable from a cell component. According to Luria (133), a latent virus could be compared to a plasmagene, depending upon the effects by which it happened to be detected. Kunkel (126) considered viruses and genes as of the same order of size. Both are composed chiefly of nucleoproteins, both mutate, and both reproduce only within living cells. Now for differences pointed out by Kunkel: viruses multiply more abundantly and much faster than genes. Viruses pass freely from cell to cell; genes move only during cell division. Viruses are autonomous, whereas genes are part of the hereditary mechanism. Gortner (63) considered the virus to be a naked nucleus, which view coincides with the writer's. This digression is a gentle reminder that, while our chief concern in the past has been with macroscopic symptoms, the area of microscopic symptoms or signs should no longer be lost sight of when one is dealing with the symptom and masking phases of plant virology.

## SYMPTOMATOLOGY

Plant virus diseases are usually classified on the basis of symptoms. The ringspot diseases are prime examples of infections having acute "shock" symptoms with strikingly distinctive ring-shape lesions which become masked after the onset symptoms have passed into chronic disease. The mosaics usually have distinctive persistent symptoms, but characteristically there are mild strains of the viruses, some of which produce masked symptoms. The symptoms of some mild mosaic viruses such as the so-called masked strain of tobacco mosaic virus in tobacco (100), are masked at all seasons. Other mosaic viruses, such as affect the raspberry (3, 4, 5) produce masked diseases seasonally. Masking is less often observed in connection with yellows-type virus diseases, which commonly are infections chronic or systemic in nature. Here, high temperature may play a part and eliminate above-ground symptoms (124, 125). Also heat will cure budstocks of certain viruses. On the other hand, the locust "witches broom" virus may persist for a long time in stems and roots of mature trees without producing obvious disease symptoms (106, 201). The same is true of citrus psorosis (43). The tobacco necrosis virus (163, 180, 182) was discovered in the roots of tobacco plants which had never shown symptoms except when inoculations were made manually on the foliage with extracts of root juices. Aggressive viruses which cause stunting, dwarfing, and rosette, and often result in death are rarely masking. Ordinarily, these diseases exhibit other signs or symptoms of internal disorder which are nonmasking because they reduce plant stature or size (*a*) by stunting the over-all growth; (*b*) by dwarfing the leaf or organ size; (*c*) by rosetting the terminals through shortening of internodes; and (*d*) by dwarfing, pigmentation, and distortion of leaves. The technical

or scientific names of viruses are also based on macroscopic host symptoms even though the masking phase obtains through most of the growing season. We are indebted to Holmes (101) for developing a natural system of nomenclature or classification and simple guide, based on symptomatology (20, 102, 103).

## MOSAIC DISEASES

The mosaics, while possibly not the most conspicuous, are probably the most important virus diseases of plants. Tobacco mosaic was the first plant disease of virus origin to be identified and recognized as such (2, 105, 142).

### Potato Mosaics

The potato mosaic virus complex probably has no equal in complexity among plant virus diseases. It ranges all the way from severe disease symptoms to complete masking or latency, or both.

In 1923, Schultz & Folsom (174), in studies on tranmission, variation, and control of certain "degeneration" diseases of Irish potatoes, reported that degeneration was brought about by at least seven virus diseases, for which the etiology remained to be demonstrated. These diseases are quite similar but differ sufficiently in mode of transmission and symptoms to be considered distinct even in the same variety and in the same environment. Insect transmissibility was proved for the causal agents of six diseases and for several of their combinations.

There was a reluctance to employ the terms "virus" and "viruses" in relation to causation or etiology of mosaic degeneration in potatoes and other crops. There was a possibility of the occurrence of protozoa in plants affected with bean mosaic (151). In 1925, Schultz & Folsom (175) extended their transmission and dissemination experiments on the virus diseases of potatoes. Three techniques—leaf mutilation, aphid feeding, and grafting—were successful for the transmission of potato viruses. In insect cages, aphid inoculation produced current-season symptoms in the Green Mountain variety with four types of mosaic-mild, leaf-rolling, rugose, and crinkle. It was Johnson (107) in 1925, who transmitted mosaic viruses to tobacco from apparently healthy potatoes. Soon this completely masking virus or viruses was designated as "latent."

Smith (177) transmitted latent potato mosaic viruses to tobacco and revealed the curious fact that needle and aphid inoculations, respectively, from the same mosaic-infected potato plant, produced characteristic symptoms in tobacco, depending upon the mode of transmission. This phenomenon was the first definite evidence of the composite nature of potato mosaics. Smith (178) presented further evidence that many potato virus diseases (mosaic, crinkle, and streak) are caused by virus combinations in nature. He developed a technique of virus isolation from a complex in the living plant by: (*a*) utilizing the selective power of virus transmission by aphids; (*b*) the use of plant indicators and plant filters, and (*c*) taking advantage of the difference in length of incubation periods and of unequal

rates of movement of constituent viruses of the complex within a tobacco plant at the moment of the development of primary symptoms in the young plant or in the aging plant. Smith described two viruses, X(latent) and Y, isolated from the potato variety Up-to-Date, and tested the reactions they cause on a selected range of host plants. He found that virus X was transmissible by needle scratch but not by aphids, whereas virus Y was transmissible by both methods. Also, virus Y was found to be an important component of virus combinations causing potato diseases of the mosaic group. It was isolated from 20 specimens having potato virus diseases of this group and was also found alone causing crinkle in several potato varieties. This discovery as to the identities of viruses X and Y was significant to investigators everywhere.

One of the confusing factors in nature is the common occurrence of virus mixtures. Furthermore, a number of viruses which alone are latent or masking, are often companions of other viruses in the same hosts. In certain virus combinations there are synergistic effects which produce severe disease.

Vanterpool (188) found the latent or X virus of potato, in combination with tobacco mosaic virus, causing a severe streak in tomato. He also repeatedly isolated a secondary yellow bacterial organism from the tomato streak lesions. Juice extract containing streak virus was subjected to several treatments for removal or elimination of the bacterial contaminant which definitely established that tomato streak virosis was not caused by bacteria. According to Koch (121), another combination of virus X with vein-banding virus, or with virus Y in potato, causes rugose mosaic.

Jones *et al.* (108) tested many combinations of potato viruses and concluded, as had other workers, that when the latent and vein-banding viruses of potatoes were combined in different ways they were able to cause such virus diseases as rugose mosaic, crinkle mosaic, mild mosaic, and several streak diseases. Using tomato and tobacco plants as indicators, they found virus X present in 1079 apparently healthy potato plants as well as in 188 potato plants affected also with various other virus diseases. From this type of experimentation there was developed a systematic certification program whereby the potato industry effectively and successfully combats the potato viruses.

Smith (180) named the viruses causing the composite mosaic diseases affecting the potato plant Solanum Viruses 1-18. He stated:

> While a certain number of viruses affecting the potato plant are widely distributed throughout the new and the old worlds, there are others which are found only in the former. The following viruses occur in Europe and North America, and are probably found wherever the potato is grown, Solanum Viruses 1, 2, 3, 4, 6, 9, 14, and 15. Certain potato viruses, however, appear to be found only in North America; these are Solanum Viruses 11, 12, 13, 16, and 17. On the other hand, one or two seem to be confined to Europe, such as Solanum Viruses 5, 7, and 8.

The question of potato viruses being harmless or masking was discussed

by Murphy (150). It appears that no virus is entirely harmless, although in some cases no adverse effect upon yield was demonstrated.

Smith (180) found that the principal potato mosaic diseases are caused by four viruses: X (which commonly is masked in the older varieties in the United States), Y, A, and F (Solanum Viruses, 1, 2, 3, and 8, respectively, alone or in combination) and these form the types of three natural groups. Solanum Viruses 2 and 3 (Y and A), although distinct, are considered together because of the occurrence of intermediate forms. The four viruses mentioned, together with their combinations, produce eight recognizable diseases named:

| | |
|---|---|
| (*a*) Solanum 1 (Virus X) | Simple mosaic |
| (*b*) Solanum 8 (Virus F) | Yellow mosaic |
| (*c*) Solanum 3 (Virus A) | Veinal mosaic |
| (*d*) Solanum 2 (Virus Y) | Veinal mosaic and leaf-drop streak |
| (*e*) Solanum 1 plus Solanum 8 | Interveinal mosaic |
| (*f*) Solanum 1 plus Solanum 3 | Crinkle |
| (*g*) Solanum 1 plus Solanum 2 | Rugose mosaic and leaf-drop streak |
| (*h*) Solanum 3 plus Solanum 8 | Double-virus aucuba mosaic |

Combinations of more than two viruses also may occur.

The first three diseases are often overlooked in the field, the eighth is not very common, and the remainder make up the "mosaic" of potato, caused by viruses transmitted by *Myzus persicae*, occurring alone or in combination with Solanum Virus 1 (virus X). This reduces the mosaic problem to two dimensions: the control of *M. persicae* and of virus X. In practice this means the production of virus-free "seed" potatoes and the production of virus-resistant or virus-tolerant potato varieties, which has been the program of action by the potato industry for the past 25 years.

Schultz, who helped pioneer the investigations on potato viruses in 1917, is still active, although officially in retirement. He avers that investigations over the past 20 years have added little that is new about potato viruses. The mosaic symptoms are all masked by high temperatures while most of the others (leaf roll, spindle tuber, witches broom, yellow dwarf) are not. Smith (181) gave the number of strains for the respective potato viruses as follows: virus X, 7; virus Y, 4; virus A, 3; and virus S, 1. The nonmosaics or yellows types are usually accentuated by high temperatures which make them more conspicuous for roguing in the seed certification program.

### Raspberry Mosaics

Smith (180), in his textbook on plant viruses, reviews the raspberry virus diseases in Europe and North America. In general, high temperatures induced masking of so-called mild mosaics of red and black raspberry. Bennett (3, 4, 5) stressed the mosaic masking symptom picture, especially on black raspberry. The effect of temperature on symptoms in black raspberry is very striking; under Michigan conditions one often finds on new canes

rings of leaves which show mottling. Ordinarily, new canes of raspberry produce leaves without symptoms, even though the plant is systemically infected. If the temperatures drop low enough for a few days, young leaves which are subjected to it and which develop later, may show mottling.

Bennett (3) observed five virus diseases of raspberry in Michigan: (*a*) curl, (*b*) red raspberry mosaic, (*c*) mild mosaic, (*d*) yellow mosaic, and (*e*) streak. All except streak, are aphid transmitted. The chief vector of mosaics is *Amphorophora rubi* and of curl is *Aphis rubiphila*. Plants show symptoms of curl after inoculation by single aphids, and aphids seem to carry the curl virus for over three weeks. Temperature has a very decided influence on the masking symptoms of mild mosaic and red raspberry mosaic. Plants with mild mosaic show no symptoms of mottling at 28°C., at which temperature the symptoms on plants affected with red raspberry mosaic are very mild. Under field conditions in Michigan, the yellow mosaic symptoms are distinct all season. The curl symptoms are masked at all temperatures at which plants will grow. More severe symptoms are characteristic of black raspberry infection.

In girdled plants, the curl virus failed to pass the obstruction; hence, the virus must move through the bark (phloem). After inoculation, curl virus moves slowly. In the raspberry virus group there are wide varietal ranges of susceptibility, apparent immunity or masking, and disease tolerance.

Bennett (4) observed two kinds of raspberry curl virus in Ohio: (*a*) one attacks only the red raspberry and (*b*) the other attacks both red and black. Both types were spread only by aphids (*Aphis rubifera*) and as far as one-half mile from the original source of infection. All known raspberries, blackberries, and dewberries are susceptible to at least one type of mosaic, of which there are four: (*a*) A mild mosaic occurs on red, purple, and black raspberries, with symptoms practically always masked on red raspberries, very mild on black varieties, and with very little stunting. (*b*) The medium-type mosaic is much more severe than mild mosaic and has a more general distribution. Symptoms (yellow and faded spots) occur on the red raspberries and only on the first leaves formed in the spring, with little noticeable injury to the plants. On the black raspberries the injury is greater, the leaves produced at low temperatures are distinctly mottled, and the plants are considerably dwarfed. Later in the season when the older leaves yellow and die, symptoms are difficult to find. Cool spells in summer may result in rings of mottled leaves on new canes. The current-season infections are usually detected regardless of summer temperatures. (*c*) A severe-type mosaic occurs on red raspberries with distinct mottling at low temperatures but masked at high temperatures. On the black raspberries it is still more severe and death results. (*d*) The fourth type, or "yellow mosaic," was considered to result from a distinct virus. It attacks and stunts all varieties and kills the black raspberries. Symptoms appear late on red raspberries, and though they are visible throughout the rest of the season they are masked somewhat by temperature effects. In Ohio, the aphid vector

(*Amphorophora rubi*) prefers to feed on the red raspberries. Because of the aphids' dislike for blackberries and dewberries, these plants are largely free of mosaics. Rankin (167) coined the name "klendusic" for the symptomless plant escapes from aphid inoculation.

Bennett (5) concluded that there were three types of virus diseases affecting the genus *Rubus:* curl, mosaic, and streak, of which curl and mosaic are widely investigated and transmitted experimentally. Streak was not transmitted and its vector is unknown. The so-called "running out" of raspberries was attributed to the mosaics. Cooley (37) demonstrated that wild brambles are often the source of viruses which infect the genus *Rubus.* For the maintenance of productive plantings it was necessary to develop a practical method to exclude the wild sources of virus. Cooley (38, 39) reduced to two the number of mosaics in eastern North America and proposed the names "Green Mosaic" for virus 1 and "Yellow Mosaic" for virus 2. His suggestion was based on the fact that the three mosaics of Bennett (5) on the red raspberries produced a single uniform expression on Columbian black raspberries.

### Dodder Latent Mosaic

Bennett (8) discovered that dodder latent mosaic virosis is masked on all species of *Cuscuta* tested and probably in many other native host plants in the area. Several species and varieties of plants, including sugar beet, celery, pokeweed, tomato, and potato showed marked disease symptoms after inoculation with this virus, but soon practically recovered from symptom expression. Subsequent inoculation with tobacco mosaic virus or tobacco etch virus conditioned the tomato plant for the reappearance of symptoms of dodder latent mosaic. Several other species and varieties including *Nicotiana glauca,* Turkish tobacco, *Brassica adpressa,* and dodder species are hosts of the virus but show no recognizable symptoms of disease. Recovery from symptom expression is accompanied by a marked reduction in virus concentration. Virus concentrations in symptomless carriers infected for more than three weeks are very low compared with those in recently infected pokeweed plants. However, in one symptomless carrier, *Cuscuta campestris,* the virus reaches a relatively high level a few days after inoculation and then recedes to a relatively low level.

When young tomato plants, which had recovered from infection with dodder latent mosaic virus, are inoculated with tobacco mosaic or tobacco etch virus, symptoms of the dodder latent mosaic reappear on the young leaves and continue to show as the plants become dwarfed. The dwarfed condition results regardless of the sequence of introduction of the virus components of the mixture. The concentration of dodder latent mosaic virus in dwarfed plants appears to be maintained at levels much higher than those in recovered plants affected with it alone. The presence of dodder latent mosaic virus appeared to have no influence on the concentration of tobacco etch or tobacco mosaic virus. It would appear that the critical

factor in recovery of plants from dodder latent mosaic has to do with masking concentrations too low to produce marked symptoms.

### *Brassica* Viruses

Broadbent (30) reported two important virus diseases of *Brassica* crops—cauliflower mosaic and cabbage black ringspot. Symptoms of cauliflower mosaic virus were more severe at temperatures below 24°C., whereas those of cabbage black ringspot virus were more severe above that temperature. The cauliflower mosaic symptoms are masked when young plants grow at temperatures above 24°C. Also, masking occurs during winter if plants are infected with milder strains. Shading delays symptoms of both diseases and diminishes their severity.

Cucumber mosaic virus occasionally infected *Brassica* spp. and one strain multiplied in cabbage, cauliflower, and brussels sprouts without causing symptoms. This virus caused a slight distortion of turnip leaves but was usually carried in most other cultivated *Brassica* species without symptoms.

### Sweet-Potato Virus Complex

Brief mention will now be made of the sweet-potato virus complex which apparently consists of two syndromes based on aphid and mechanical transmission of the virus associated with internal cork (90), and natural plus graft transmission of the feathery mottle virus. The incubation period of internal cork virus syndrome was reduced from about one year to one week by using Scarlett O'Hara morning glory (89). However, after the initial shock symptoms are produced, whether on the sweet-potato or indicator plant, there was masking.

The rapid transmission technique through several modifications was found applicable to both virus syndromes. Also, in addition to the morning glory, we now have a sweet-potato seedling indicator clone (Louisiana VII) in which these viruses have an incubation period of less than two weeks (91).

### Ringspot Diseases

Holmes (102) described the ringspot group as viruses causing diseases characterized by necrotic or chlorotic spotting with concentric-ring lesions, and "eventual recovery with nonsterile immunity." Bennett (8) pointed out the complete or nearly complete recovery from primary symptoms of certain virus diseases, including ringspot of tobacco and curly top of tobacco and tomato.

### Tobacco Ringspot

The tobacco ringspot disease was first discovered in Virginia by Wingard (204). Apparently this virus is more widely distributed in nature than was first thought. The fact that it is seed-transmitted by petunia is significant (65).

Price (161) described the recovery of several species of *Nicotiana* from

tobacco ringspot as a normal behavior, which was distinguished from exceptional thermal masking because it occurred under most environmental conditions, and recovered plants could not be induced to develop symptoms by reinoculation. This phenomenon of recovery and subsequent failure to develop symptoms on reinoculation was referred to as "acquired immunity," which is not accompanied by complete disappearance of virus.

When there is virus retention by the plants the term "acquired immunity" is objected to by several workers. Smith (178) prefers the term "acquired tolerance" as also does Kohler (122). Valleau (191) considers that there is actually no recovery, but a stage of disease when the leaves are parasitized to the limit and patternless. Limited studies by the writer indicate that there is a devitalization effect which accompanies the nonsterile immunity conferred by presence of the virus in symptomless tobacco, and this effect should favor retention of the "acquired immunity" terminology suggested by Price (162).

McKinney & Clayton (138) clearly defined acute and chronic symptoms in the tobacco ringspot disease. In the short, first stage, the symptoms were pronounced and in the long, second masking stage, symptoms were much less prominent as is characteristic of several other virus diseases.

### Tomato Ringspot

Price (162) first obtained tobacco ringspot No. 2, or tomato ringspot, virus from plants in an otherwise healthy set of Turkish tobacco in a greenhouse at Princeton, New Jersey. It was shown by the immune reaction to be entirely distinct from ordinary tobacco ringspot No. 1 and from green and yellow ringspot. Tobacco plants infected with tomato ringspot recover and develop a solid immunity to the disease but do not become immune to tobacco ringspot No. 1 or to any of the eleven other virus diseases tested.

In the early spring of 1940 in the greenhouse at the Rockefeller Institute in Princeton, the writer encountered the same concentric-ring contaminant in some otherwise healthy Turkish tobacco plants. This finding was fortuitous because the tomato ringspot virus had just been obtained from red currants afflicted with severe currant mosaic (66) in the Hudson Valley of New York State and also locally in a currant planting on the Lambert Estates. Hitherto, the ringspot symptoms had not been detected on currant with mosaic symptoms because of masking (69). Currant mosaic symptoms were completely masked at 70°F. and above. Thus, the ringspot symptoms were first observed on currant in a greenhouse under high temperature conditions (75° to 80°F.) which completely masked currant mosaic. It should be noted that the concentric-ring patterns were present only on occasional young unfolding leaves and that these lesions became masked as the leaves matured. The currant plants affected with both mosaic and ringspot viruses again exhibited typical mosaic symptoms in outdoor plantings in 1941 (71).

An endeavor to find the native virus source other than the red currant

was unsuccessful. However, another visit to the Lambert Estates currant planting revealed a weed with dark-green ringspots. The identity of the causal virus was not determined, but in tobacco the virus did fail to protect against tobacco ringspot and currant or tomato ringspot.

Incidentally, in this study all three ringspot viruses were incorporated into individual tobacco plants through successive inoculations of rooted cuttings from recovered, symptomless plants. By the time the third virus was incorporated the triple-infected tobacco plants were so weak and devitalized that their cuttings failed to root and died apparently from the cumulative additive virus effects.

## SUGAR BEET CURLY TOP

Curly top symptoms are masked in a number of host species. Perhaps the most striking masking occurs in *Nicotiana glauca*. This species is susceptible to most of the virulent strains of curly top virus but shows no symptoms. Under natural conditions many plant species are infected with curly top. Holmes (102) lists 41 plant families of which 19 contain species susceptible to curly top virus.

Bennett (6) demonstrated the internal tissue relations of the sugar beet curly top virus in relation to masking and symptom expression. His illustrations on pathological histology and on how the beet leafhopper feeds are classics. The vector *Eutettix tenellus* (currently *Circulifer tenellus*), feeds on the leaf veins and its mouth parts usually penetrate the phloem region. As the mouth parts are inserted the insect lays down a gelatinous sheath which completely encases the stylets and thereby protects the virus from external exposure.

Bennett (9) reported recovery and masking of curly top virus in water pimpernel (*Samolus parviflorus*) and the reactions of recovered plants to reinoculation with different virus strains. Strains of curly top virus of different degrees of virulence produced reactions differing widely in severity on water pimpernel. Symptoms ranged from vein clearing, slight leaf rolling, and little or no stunting produced by the less virulent strains, to severe curling, distortion, yellowing, and necrosis of leaves and death of a part or all of the affected plant produced by the more virulent strains. In plants which survived the early effects of the disease, recovery from symptoms was complete or nearly so. While plants recovered from the more virulent strains were somewhat less vigorous than healthy plants, there was no evidence that the virus strains were attenuated or otherwise modified. Recovery was associated with a marked reduction of virus concentration. Transmission of virulent strains from recovered plants by means of dodder (*Cuscuta californica*) resulted in production of severe symptoms of disease in the inoculated plants, indicating that no protective principle was transported from recovered to healthy plants. Plants recovered from one strain were susceptible to further infection by any other more virulent strain. Plants recovered from strains of medium to high virulence

were sometimes protected against the full effects of strains of still greater virulence.

Complete or nearly complete recovery from primary symptoms was reported for curly top of tobacco by Bennett & Esau (11) and by Wallace (195); and acquired immunity from curly top in tobacco and tomato by Wallace (197). Wallace (197) listed the plant reactions to curly top virus and from this he concluded that these phenomena are immunologic in nature.

The sugar beet curly top virus is characterized by strains which can be modified by plant passage. Lackey (127, 128) showed that passage through lamb's-quarters (*Chenopodium album*), tomato, squash, watermelon, spinach, and resistant varieties of sugar beets, gave attenuated or partially masking strains of curly top virus. Passage of mild strains through the highly susceptible weed, *Stellaria media,* restored virulence (129, 130). In further work, he confirmed and extended these findings (131). Giddings (54) distinguished four different strains by their ability to produce several degrees of injury on different host plants. Later (55) he isolated six other new strains of virus, and found (56) that the rate of spread in sugar beet fields depended upon the strain.

Bennett (7) showed a correlation between movement of the curly top virus and translocation of food in tobacco and sugar beet. Wallace (196) presented evidence of passive immunization of tobacco against the curly top virus.

Wallace (198), in discussing the immunological properties of "cross-protection" and "acquired immunity" of the plants which recover from curly top virus, contended that they differ from those reported in other studies and more nearly approach true immunological reactions. In brief, Turkish tobacco plants recovered from severe symptoms were resistant to reinoculation, and contained virus unchanged in virulence. Healthy tobacco plants, inoculated by grafting with scions from recovered tobacco plants, developed mild symptoms. Healthy tomato plants developed severe symptoms when inoculated by means of insect transfer from recovered tobacco, but developed mild symptoms when inoculated by grafting with recovered tobacco. The mildly affected tomato plants contained virulent virus and had a certain degree of protection from subsequent inoculations. The "acquired immunity" or "degree of protection" in tomato plants, which varies with different virus strains, was retained through many vegetative generations. The degree of protection based on cross-inoculation with twelve strains of the virus, ranged from very slight to complete.

## CITRUS TRISTEZA

Tristeza symptoms are largely masked in most varieties of citrus except when occurring on the sour orange rootstock. The scion-rootstock incompatibility of sweet orange on sour orange prevalent in South Africa in 1924, soon appeared in Ceylon, West Africa (104), Australia, Java, South

America, New Zealand, and North America, or so it seemed. Thus, by 1944 the tristeza disease was destroying the citrus industry of South America and was a threat in California where it was called "quick decline."

The work of Bennett & Costa (10) in addition to the work of others, delineated the nature and control of this disease and served as the basis for certification of tolerant rootstocks (Cleopatra mandarin hybrids, Rangpur lime, sweet orange, trifoliate orange, citrunelos, citranges, rough lemon, and tangelos) for propagating sweet orange and other citrus. As work was progressing on this basis, according to Wallace *et al.* (200), the tristeza virus origin was traced back to the Orient instead of to South Africa. Olson & McDonald (154) reported tristeza virus masked in 17 out of 26 selections of Satsuma oranges in Texas. Olson & Sleeth (155) found tristeza virus carried by the Meyer lemon in Texas. Also, Wallace & Drake (199) found tristeza virus masked in Meyer lemon in California. The Meyer lemon, an ornamental, was brought to this country from China in 1908. The native home of the Satsuma orange is also Asian. Finding tristeza virus masked in oriental materials in Texas and California is strong evidence that these varieties were carriers of the same virus in their native home.

Apparently sweet oranges on sour orange rootstocks are easily inoculated with tristeza virus by means of the oriental black citrus aphid (*Aphis citricidus*). In susceptible citrus the explanation for variability in severity has been found to be a wide range of virus strains, the mildest of which protect against severe ones. Oberholzer *et al.* (152) reported that stem pitting decline of grapefruit in South Africa is also caused by the tristeza virus. With masking playing such a large role in the tristeza disease the health status of propagating materials becomes ever more important to the citrus industry in the years ahead.

The tristeza of citrus is caused by a masking virus characterized by failure to produce primary symptoms on the foliage of any known host plant and by severe injury only on trees from certain scion-stock combinations. Neither component of the best-known combination (sweet orange on sour orange) affected with tristeza is known to suffer injury when on its own roots, nor is injury evident in the reciprocal combination (sour orange on sweet orange). Similar scion-stock relations are known also in other horticultural plants.

## SCION-STOCK MASKED VIRUS DISORDERS

Many scion-stock incompatibilities have been encountered over the years and little has been done about it in certain plants. Several instances will now be mentioned briefly.

### Cherry Rootstock Disorder

Rawlins & Parker (168) made observations on the influence of rootstocks on the susceptibility of the sweet cherry to the virus causing buck-

skin. Apparently, sweet cherry trees on Mahaleb rootstocks resist, mask, or escape natural infection. However, the trees on this rootstock while apparently insusceptible may become very chlorotic after being grafted with diseased Napoleon scions. Very few sweet cherry trees on Morello rootstock become diseased. However, the trees on this rootstock are very susceptible and when infected exhibit symptoms indistinguishable from those on Mazzard stock. Napoleon scions on Mazzard and Morello stocks show little or no chlorosis but produce conical fruits which shrivel just before maturity and have abnormally short pedicels. Apparently, the buckskin virus does not readily pass through a Mahaleb stem but when it does it is largely masked.

### Disorders of Grafts on Potato Seedling No. 41956

Raleigh (166) reported an abnormal graft reaction in potato resulting from a virus infection of a scion on a resistant stock. Green Mountain scions inoculated with potato latent mosaic (virus X) developed aerial tubers, rolling of leaves, and marked stunting when grafted on healthy U. S. D. A. Seedling No. 41956, which is resistant to latent mosaic. Without virus X neither the Green Mountain variety nor its seedlings developed the abnormality described when grafted on healthy No. 41956. Thus far, Seedling No. 41956 has not been systemically infected with the potato virus.

### Apple Rootstock Disorder

Gardner *et al.* (53) reported the lethal effects of scions from certain apple varieties on Spy 227 understock. It appears that apple scions from certain sources carry a principle, or virus, which is highly toxic (or infectious) to Spy 227 roots. A single bud or a small quantity of bark in grafting carries enough lethal principle or virus to kill the Spy 227 roots. Also the suspected virus seems to multiply and spread through the tissues. Many varieties may be carriers.

### Plum Rootstock Disorder

Brase & Parker (18) attributed the decline of Stanley prune trees to the incompatibility with the Myrobalan rootstock. Apparently, the rootstock which is compatible on other plums is constricted and may be killed when the scion is of the Stanley variety.

## STONE FRUIT VIRUSES

The writer's first contact with masking in plant viruses was in a seminar in the autumn of 1929. Duggar gave a technical presentation on the nature and properties of the tobacco mosaic virus entity and cited a published report (42). Johnson discussed symptomatology and cited his report (107) on virus transmission from apparently healthy potatoes.

In 1932, as fruit pathologist, this reviewer undertook a basic study to unravel the degeneration condition called "winter injury" then prevalent

especially in stone fruit orchards in the State of New York. Visits were made to fruit-growing localities the first year and maps were made of a few orchards representative of the so-called winter-injury degeneration condition. As visits continued, the symptom-masking picture unfolded so that by the end of five years the virus sources were traced to the nurseries (75, 76, 79, 84, 87) and found graft-transmissible. By the end of a decade of study, the number of known stone fruit viroses in North America had increased from five to about 50 (92).

The woody-plant viruses presented many difficulties from the start because grafting was ordinarily the only technique available for demonstrating transmission, which usually entailed two growing seasons to complete one experiment. Smith (177) obtained a solution for the latent potato virus masking difficulty by finding that tobacco was a good indicator plant for it. His outstanding work (180) benefited investigators everywhere by stimulating the use of indicator plants.

A brief article was published on the importance of the masking virus diseases to the stone fruit industry (79). Some of the problems facing the stone fruit industry were listed: (*a*) production of certified disease-free varieties; (*b*) production of certified disease-free rootstocks; (*c*) improvement in experimental techniques; (*d*) discovery of the insect vectors; (*e*) discovery of better experimental plants; and (*f*) improved control practices. Another decade of research supported by the interstate research program resulted in the Agriculture Handbook 10 (1), an outstanding accomplishment for the benefit of the stone fruit industry.

Hildebrand (70) showed that the rapid transmission technique usually shortens the incubation period from a year to a month or less, and listed the four chief obstructions to progress faced by investigators of woody-plant viruses: (*a*) the long incubation periods which obtain when ordinary transmission techniques were employed; (*b*) the lack of adequate details of how these viruses moved in their respective hosts; (*c*) the almost complete lack of knowledge of the identity of the insect vectors; and (*d*) the lack of adequate attention to the possibility of developing new transmission and indexing techniques.

The masking tendency or absence of recognizable symptoms, whether caused by long incubation periods, insusceptibility, latency, or high-temperature masking (hiding) of the virus entity, characterizes many of the woody-plant viruses. The writer's first encounter with latency or total masking in stone fruits was the finding that prune dwarf virus was frequently present in apparently healthy Damson and Bradshaw plums (73).

## Plum Virus Complex

The plum virus complex was a puzzle from the beginning to the end of the writer's studies covering twelve and one-half years in New York State. The principal rootstock (Myrobalan plum) from all sources was found to be affected with "asteroid spot" (80) or "chlorotic fleck" (18, 19). Re-

gardless of the source, several plum varieties, including Abundance and Red June, were carriers and maskers of a line-pattern virosis (32) which was symptomless at temperatures above 70°F. A marginal leaf etching or burn on Italian prune confused the symptom picture until its physiogenic nature (potash deficiency) was diagnosed and eliminated by fertilization.

*Prune dwarf.*—After passing through the incubation period the prune dwarf virus produced striking symptoms (187) on the Fellenberg variety of Italian prune. However, this same virus was masked completely or was latent in the Damson and Bradshaw plums (73). Berkeley (12) reported a severe outbreak of prune dwarf in Ontario, Canada, where a Damson plum orchard had been top-worked to Italian prune. When Italian prune trees were included in the indexing plantings, symptoms simulating prune dwarf were located in the cherry virus complex (77). In a recent study, Cameron & Moore (31) performed tests to distinguish the sour cherry viruses from prune dwarf virus.

Berkeley & Willison (16) tested 14 suspect viroses on seven indexing hosts in the field. Two viruses from Eagle and Elkhorn sweet cherries were shown as strains of prune dwarf. Sour cherry yellows virus is apparently closely related to the prune dwarf virus because typical strap-shape leaves developed on Italian prune and Lombard plum from some sources. These authors (17) noted that the acute symptom phase of cherry yellows was very similar to necrotic ringspot, but that the chronic-symptom phase on peach and plum included symptoms simulating those of prune dwarf.

Cochran (36), in an indexing test, induced prune dwarf symptoms on the Italian, Giant, Standard, Emilie, President, and Tragedy varieties (all *Prunus domestica*) but failed on Becky Smith, Red June, Abundance, Burbank, Flaming, Delicious, Santa Rosa, Beauty, Wickson, Eldorado, Mariposa, Mottley, Green Gage, Elephant Heart, and Apex (all *P. salicina*). The Tragedy prune was most severely affected and should be a better indicator plant than Italian prune.

*Lombard latent virus.*—Although the Lombard plum variety was moderately susceptible to the prune dwarf virus, it was found to be a symptomless carrier of Lombard plum latent virus (74), which produced a necrotic leaf spot on Italian prune (87). Foilage symptoms of Lombard plum latent virus on Stanley prune are similar.

*Shiro line-pattern virus.*—Berkeley (12) reported an outbreak of Shiro line-pattern mosaic in Canada. It appeared in an orchard of Firsts plums, the symptomless carrier which had been top-grafted to Shiro plum. Gilmer (58) demonstrated the probably coidentity of Shiro line-pattern and apple mosaic viruses. As the season advanced and temperatures rose, the symptoms on newly-formed leaves of apple and plum became increasingly masked and above 90°F., were completely masked.

*Myrobalan asteroid spot.*—An "asteroid spot" abnormality, presumably of virus nature, commonly occurs on Myrobalan plum seedlings in New York (83). Every collection brought together over a five-year period always

had some spot lesions when grown outdoors [(87) Fig. 38]. All seedlings grown indoors in the greenhouse were symptomless. Brase & Parker (18, 19) confirmed these observations and presented evidence also that the affected stock supports reduced growth of the budlings of plums and prunes.

### Cherry Virus Complex

Except for short periods of symptom expression, depending upon the environment, the cherry virus complex is almost completely masking. Stewart (184) was the first to record the prevalence of a yellow leaf drop on the Montmorency cherry in New York. Cherry yellows went by such names as "yellow leaf drop," and "physiological yellow leaf" (87). Confusion as to its cause arose because of the tendency of the symptom pattern to vary in amount and in time of incidence from season to season.

In 1932, this complex in New York went by the name "winter injury." In Wisconsin, it was called "boarder tree." A tissue discoloration was associated with the devitalization from which many trees were suffering. Attempts to correct low yields of affected trees by drastic pruning and extra fertilization proved fruitless (82). However, there was a marginal leaf etching or burn condition which was corrected by addition of KCl to the soil. Failure to control fungus leaf drop altered the growth condition of the trees and also masked virus symptoms (81). In fact, the earlier nursery mappings on cherries were often valueless when heavy fungus defoliation weakened the trees and obscured or completely masked virus symptoms. Thus, a successful fungus control program had to be worked out with the nurserymen and adhered to before virus symptoms could be recognized and the nursery plantings could be certified as virus-free.

Changes in the temperature levels from year to year altered both the time of appearance and severity of cherry yellows symptoms (94, 146). Gloyer & Glasgow (62) reported as high as 60 per cent yellow leaf drop. Some Montmorency cherry trees showing 50 per cent leaf drop in June of one year had less than 10 per cent defoliation the following year. The time of first appearance ranged from June 5 to July 6 over the years and rarely was the period of defoliation as long as two weeks.

Ringspot symptoms occasionally appear when the first leaves are unfolding in May. By the time yellows symptoms appear in June, the ringspot symptoms are so obscured by the later-formed masked growth as to be unobservable.

The source of the cherry virus complex was traced to the nurseries in 1936 (87). One variety of sour cherry (Chase) was found to be practically 100 per cent diseased in one nursery and its propagation was abandoned permanently. Within a short time it was found that the severe outbreaks in nurseries could be traced to budstock material taken from orchard sources. Even when nurseries used their own varietal material for propagation, there remained from two to five per cent yellows infection in their

plantings, and this virus contamination was traced to the rootstock source (84).

In an attempt to clarify the cherry virus complex, two phases of the experimental approach received special attention: the search for indicator plants and the development of improved transmission techniques. This combined approach led to the development of rapid transmission techniques through adjustment of the growth status and use of virus-free indicator plants (67, 70, 72).

The only virus-free stone fruits suitable as indicator plants for indexing the stone fruit viruses were peach seedlings, since none of the cherry (Mahaleb or Mazzard) or plum (Myrobalan) seedlings were found consistently healthy. Cation (33) demonstrated that both Mahaleb and Montmorency seeds carried the ringspot virus and Mahaleb seeds also carried the yellows virus. Mahaleb grafts made on peach seedlings in the field indicated over 20 per cent transmission with about half showing typical rosette (cherry yellows). Similar inoculations with Montmorency seedlings resulted in more than 20 per cent disease transmission, but only the ringspot virus was transmitted through Montmorency seed. At least 10 per cent of the Mahaleb seed carried or transmitted the ringspot virus and at least 8.7 per cent transmitted the cherry yellows. The first report on the seedborne nature of the so-called sweet cherry tatter-leaf or ringspot virus disease of Mazzard cherry was made by Hildebrand (84). Cochran (35) reported passage of the ringspot virus through Mazzard cherry seeds. Of 467 seedlings which grew, 25 or 5.4 per cent developed symptoms. Gilmer (57) found that Mahaleb cherry seeds imported from France were carriers of the necrotic ringspot virus.

The masking cherry virus complex has been studied extensively for the past 20 years (1, 92). Some of the reports were made by Hildebrand (81, 82, 87); Berkeley & Willison (16, 17); Mills (146); Willison (202); Willison *et al.* (203); Posnette & Cropley (159); Posnette & Ellenberger (160); Reeves & Cheney (169, 170); Hobart *et al.* (97); Fulton (51); and Milbrath (144). Graft transmission of cherry yellows was first reported by Keitt & Clayton (109 to 112). Temperature as a masking factor was greatly clarified when Keitt & Moore (113) demonstrated that temperatures of 20°C. and above always masked cherry yellows, whereas the yellow leaf symptoms developed freely at 16°C. under controlled conditions. Also, the low-night, high-day diurnal temperature effect was clarified.

### Peach Virus Complex

Peach virus diseases as a group were the first of the stone fruit viroses to be studied. The chief obstacle when working with the newer peach viruses was not true masking but the extended incubation periods of a year or longer under field conditions. Practically all of these viruses have yielded to the use of the rapid transmission techniques (86).

For example, in X-disease of peaches, by means of the rapid transmission technique (67), the incubation period of one year or longer was reduced to about three weeks, with corresponding reductions for most of the other peach viruses (86). This technique also aided in the discovery of the first leafhopper vector of X-disease (*Scaphytopius acutus* Say), (86). Peach trees affected with X-disease virus usually show no symptoms until about mid-June of the year following inoculation, when the leafspot symptoms begin to appear. Afterward, many affected leaves drop to the ground, thus masking the disease by defoliation.

In 1948, Palmiter & Parker (156, 157) reported yellow-red virosis, or X-disease, on sour cherry in New York. That the Western "X" strain of this virus could be transmitted to sour cherry in Utah was reported earlier by Richards (171), and Richards *et al.* (172, 173).

A new case of peach rosette mosaic (a Michigan disease) was observed in a fruit nursery in New York in 1940 (69) and eradicated. The mosaic symptoms gradually masked as the season advanced and air temperatures increased, whereas the wavy leaf character and rosetting of foliage, and stunting of trees persisted.

## INDEXING HOSTS FOR STONE FRUIT VIRUSES

Numerous investigators have made important contributions toward the understanding of indicator plants or indexing hosts. The writer (67, 70) developed the rapid transmission technique which involved adjustment of the growth status of peach seedlings for immediate virus reception in the new growth. Using this technique, it was possible (72) to index the cherry yellows complex on peach with the production of symptoms consisting of ringspot and rosette for yellows virus and dieback and recovery for ringspot virus. By this method nursery propagating materials could be indexed at any time during the year. Also by this method, virus contamination was readily detected and eliminated from the scion source. This indexing work became part of the New York State control program, in which growers, nurserymen, inspectors, and the college participated (72, 75, 76, 84). Hildebrand (77) distinguished two strains of the yellows virus. Hildebrand & Curtis (93) employed a shading technique for inducing peach X-disease virus symptoms in mature as well as in growing peach leaves within four to six weeks after darkening. Shading was also one of the techniques used for localizing viruses for prompt expression and intensification of disease symptoms (78).

Although successfully transmitting peach rosette and X-disease viruses from peach to tomato by means of the dodder vector, Hildebrand (85) was unable to transmit cherry viruses to any herbaceous plants tested, including cucumber, by this means. Moore *et al.* (147) discovered a new indicator plant when they mechanically transmitted to cucumber a virus from sour cherry. Moore & Keitt (148) used the same method in indexing sour cherry for both ringspot and yellows. Fink (44, 45), in a comparative study with

*Prunus cerasus* and *P. persica,* found *P. tomentosa* more suitable as an index plant for the cherry viruses capable of inducing necrotic ringspot. Fridlund (47) found *P. tomentosa* a satisfactory index plant for necrotic ringspot virus in comparison with Montmorency sour cherry, peach, and the Kwanzan and Shirofugen varieties of flowering cherry (*P. serrulata*).

Fulton (49, 50) tested four cherry viruses transmitted mechanically from sour cherry to 227 test species of 18 families grown in pots in the greenhouse. He found that all viruses (A, B, E, G) produced similar symptoms on cucumber but induced numerous symptom differences on other hosts. In these findings the four viruses appeared to be distinct. Mulder (149), working in Germany, also transmitted mechanically a sour cherry virus to cucumber. Gilmer (59) studied 123 isolates from *Prunus* species, and two isolates from *Rosa multiflora* on cucumber plants and found all virus extracts infective in the pH range 4.2 to 11.6, and all irreversibly inactivated at pH 3.8 and pH 12.0, with the thermal inactivation points practically identical.

Hobart & Buchholtz (96) suggested that a good indexing host for cherry yellows should express distinct symptoms when first infected with virus and before masking. Their experiments point to failure of *Prunus mahaleb* to express symptoms reliably at high greenhouse temperatures and to lack of virus potency in it at temperatures favorable to symptom expression. *P. tomentosa* reliably expressed symptoms when budded with virus-infected *P. mahaleb* and sour cherry buds at the three greenhouse temperatures (60°, 70°, 80°F.). However, *P. mahaleb* reliably expressed virus symptoms only at 60°F. Hitchborn (95) obtained results which confirm the idea that different strains of the same virus may behave differently in plants at high greenhouse temperatures.

Gilmer & Brase (61) studied the comparative value of six *Prunus* spp. as indexing hosts in detecting stone fruit viruses. In the greenhouse, peach proved inferior and Nanking and Shiro plums were intermediate. The most efficient were Albion plum and Montmorency cherry and, in the field, Shirofugen flowering cherry, but each of these gave 10 to 15 per cent false negative indices. National Pickling cucumber inoculated at the cotyledon stage with leaf sap from budded peach, reacted positively to all isolations, including three which had not produced symptoms on any of the *Prunus* species.

Milbrath (143) found Buttercup squash a good differential host for strains of stone fruit viruses, producing bright golden patterns. The same virus strains developed distinct local lesions on Hubbard squash. In this study 25 virus cultures were established on squash. The indexing sequence was to place virus buds from source trees into peach, then transfer from peach to cucumber and then to squash. Virus from 780 cherry sources caused bright golden yellow leaf symptoms, whereas virus from other stone fruit sources developed only local lesions, mild chlorotic mottle, severe growing point necrosis, or no symptoms at all. Gilmer (60) found a "latent" virus

in Montmorency cherry from three sources. It produced both A (severe) and B (mild) symptoms and was rapidly detected by inoculation of Cocozelle squash seedlings which developed vein-clearing symptoms indistinguishable from those of other stone fruit viruses. It is possibly Shiro plum line-pattern virus.

## VIROSES OF ORNAMENTALS

### Chrysanthemum Stunt

Only recently have indicator plants been used in studies on the virus diseases of ornamentals. Brierley (22) reported that inoculation of 76 species and varieties of plants, largely of the family Compositae, revealed 39 susceptible and 37 insusceptible to stunt. Of the susceptible kinds, only seven developed recognizable symptoms, five of which were species or varieties of the genus *Chrysanthemum* and two species of *Senecio*. The others could serve as masked symptomless carriers of stunt virus when naturally infected. No other test plants were found equal to the Mistletoe and Blazing Gold chrysanthemum varieties as indicators for stunt. No species of chrysanthemum tested was to be found immune from stunt, but *Chrysanthemum parthenium* and *Tanacetum vulgare* showed resistance.

### Other Chrysanthemum Diseases

Brierley (23) found Blazing Gold a good indicator plant for stunt and rosette viruses, and Mistletone a good indicator for mosaic and stunt but slow to respond to rosette virus. The Good News variety gives clear reactions to mosaic and rosette viruses and distinguishes them. It expresses stunt as transitory yellow veining. Chrysanthemums are reservoirs for tomato aspermy virus, which also affects many other plants including tobacco. This virus is carried by the green peach aphid (*M. persicae*).

The four aphid species which transmit aspermy virus also transmit B virus from chrysanthemum to chrysanthemum. The B viruses or mosaics are common on chrysanthemum in the United States and of the 8 known, 6 go to petunia but not to tobacco. When grafted into the Nightingale variety of chrysanthemum, known to carry both aspermy and B viruses, the flower distortion described by European workers failed to develop. Most varieties which expressed B virus mottling or vein clearing symptoms in young, actively growing leaves became masked with age. A few varieties never showed clear symptoms. Leaf symptoms were generally masked as chrysanthemums approached flowering. The Good News variety expressed well-defined Virus B mottling without leaf distortion.

### Rose Virus Diseases

Brierley (24) discussed the viruses on roses. Fulton (48) transmitted rose mosaic virus mechanically from rose to cucumber and cowpea. From the cowpea he transmitted it to 25 other species in seven plant families. As with many other ornamentals, rose understocks and varieties, generally, mask viruses

### Miscellaneous Virus Diseases

Brierley (25), in discussing virus diseases of ornamentals, concluded that viruses occur infrequently in the seed, are often insect-transmitted, with the true carriers often masked or unknown. Keur (116) reported studies on the occurrence, transmission, and appearance of virus diseases in the genus *Abutilon*.

Brierley (26, 27) found florists' hydrangea affected with the masking tomato ringspot and another unidentified virus, and reported hydrangea ringspot virus to be the cause of "running out" of the florists' hydrangea. Bridgmon & Walker (21) reported gladiolus as the masked reservoir of cucumber mosaic, tobacco ringspot, and bean-yellow mosaic viruses. Brierley *et al.* (29) confirmed their report. McWhorter & Brierley (140) reported that most virus diseases are masked and undetectable in lily seedlings before the second and third seasons of growth. Travis & Brierley (190) isolated one strain of tobacco virus from iris and another from easter lily. No symptoms were observed in naturally infected plants.

## HOP VIRUS DISEASES

Keyworth & Paine (119) listed six possible virus diseases of hops in England. Keyworth (117) observed nettlehead and mosaic as important masking hop virus diseases in England. In warm weather, the vines may be normal in appearance, rendering positive identification of nettlehead difficult. Adjacent plants may acquire infection but remain symptomless for the remainder of the season. Some varieties became tolerant to mosaic and masked this virus as the season advanced and thus carried the disease without showing symptoms. The virus symptoms were always suppressed in warm weather in the greenhouse (118).

## VIRUS DISEASES OF CEREALS AND GRASSES

The cereal and grass viruses form a complex not unlike that found in potatoes and stone fruits, for there are reactions or expressions ranging from symptomless to severe symptoms.

McKinney (136) described Atsel barley as an indicator plant for wheat streak and several other mosaic viruses. The local lesions for wheat streak are large, yellow, and frequently fusiform. Atsel barley is highly susceptible to the viruses of brome mosaic and barley stripe mosaic. In some barleys, strains of barley stripe mosaic produce reactions from symptomless through intermediate to lethal, under a constant environment.

McKinney (137) demonstrated the maintenance of naturally infectious cultures of soil-borne wheat mosaic and oat mosaic without the use of soil. Naturally infective cultures are maintained in washed, autoclaved river sand by the introduction of thoroughly washed roots from mosaic plants.

McKinney and his colleagues (139) demonstrated how field soils, contaminated with the viruses of wheat and oat mosaics, were easily rendered noninfectious by treatment with formaldehyde, chloropicrin, carbon disul-

phide, D-D (dichloropropenes), or ethyl alcohol. In rendering field soils noninfectious by these chemical treatments, it appears that two factors may have been dispensed with, the virus and its vector.

## STRAWBERRY VIRUSES

Plakidas (158) recognized seven distinct virus diseases of strawberry. Of these only Yellows and Crinkle were considered to be of great economic importance. Taken individually, single viruses apparently are latent in all commercial varieties with one exception, the multiplier plant virus. When symptoms ranging from severe to masking are encountered they may be accounted for by the multiple virus combinations which occur in nature. Darrow *et al.* (41) found that with all varieties susceptible to and tolerant of virus invasion the only practical control measure open to use is virus-free plants.

Harris & King (64) discovered *Fragaria vesca* as an indicator plant for strawberry viruses. Subsequently, Frazier (46) found a latent virus in the East Malling clone of *F. vesca* and developed an *EM* × Alpine *F. vesca* hybrid free of this contaminant.

## RESERVOIRS OF MASKED VIRUSES

Most investigators have attempted to establish the relation of reservoirs of masked viruses in wild and cultivated plants to virus diseases in cultivated plants. Manns & Manns (141) established wild plums as symptomless reservoirs of yellows and little peach viruses. Hockey (98, 99) reported three partially masking apple virus diseases: false sting, flat limb, and mosaic. Brierly & Smith (28) found that perennial tree onion a masked carrier of onion yellow dwarf virus. Ocfemia (153) observed the rapid dissemination of abaca mosaic when in proximity to corn. Cruz (40) made a comparative study of mosaicked and nonmosaicked fibers of abaca, the Manila hemp plant. Keller (114) recovered a new masked virus in apparently healthy Blanche chrysanthemums in combination with stunt virus. Knorr (120) described indicators and filters of citrus tristeza virus. Machinnon (134) found that green peach aphids reared on symptomless turnip stecklings acquired a virus which was transmitted to plants of *Physalis floridana* and *Physalis pubescens*, hosts of potato leafroll virus. Millikan & Guengerich (145) indexed on *Amelanchier* an agent causing a masking apple disorder. Berkeley (13) summarized the reactions of plants to virus infection including masking and latency. MacClement & Richards (135), in search of hidden viruses in the Royal Botanical Gardens in Hamilton, Ontario, Canada, reported that about 10 per cent of the plants, per se, were carrying mechanically transmissible viruses and concluded that very few wild species are free from possible virus infection.

## CONCLUDING REMARKS

Four important lines of investigation have led to the success which has

been achieved in the study of masked plant viruses; namely, (*a*) the technique of inoculation or infection; (*b*) the indicator plants or indexing hosts; (*c*) the natural vector; and (*d*) the rapid transmission techniques. Often, slow progress has been caused by virus masking brought about by (*a*) long incubation periods when poor indexing hosts and slow transmission techniques were used; (*b*) virus hiding in obscurity through lack of temperature or environment control; and (*c*) latency in certain hosts.

For the severe virus diseases, natural transmission is often biological; that is, the insect puncture or perforation feeding mechanism is involved. To clarify the role played by masking in the virus disease syndrome, the nature and biology of the vector must be determined (86, 88).

Cochran & Chidester (34) raise the possibility that the component of virulence may exist in latent form in the susceptible plant. However, such a possibility does not as yet have general acceptance among virus workers.

## LITERATURE CITED


1. Anon., *Virus Diseases and Other Disorders with Viruslike Symptoms of Stone Fruits in North America* (U.S. Dept. Agr. Handb. 10, 276 pp., 1951)
2. Beijerinck, M. W., *Koninkl. Ned. Akad. Wetenschap. Proc.*, II, **6**, 3–22 (1898)
3. Bennett, C. W., *Mich. State Univ. Agr. Expt. Sta. Tech. Bull., No. 80*, 1–38 (1927)
4. Bennett, C. W., *Ohio Agr. Expt. Sta. Research Bull. No. 454*, 43–66 (1930)
5. Bennett, C. W., *Mich. State Univ. Agr. Expt. Sta. Tech. Bull., No. 125*, 1–32 (1932)
6. Bennett, C. W., *J. Agr. Research*, **48**, 685–87 (1934)
7. Bennett, C. W., *J. Agr. Research*, **54**, 492–507 (1937)
8. Bennett, C. W., *Phytopathology*, **39**, 637–46 (1949)
9. Bennett, C. W., *Phytopathology*, **45**, 531–36 (1955)
10. Bennett, C. W., and Costa, A. S., *J. Agr. Research*, **78**, 207–37 (1949)
11. Bennett, C. W., and Esau, K., *J. Agr. Research*, **53**, 599–620 (1936)
12. Berkeley, G. H., *Dominion Dept. Agr. Publ., No. 679*, 1–3 (1941)
13. Berkeley, G. H., *Quebec Soc. Protection of Plants, 34th Ann. Rpt.*, 101–15 (1952)
14. Berkeley, G. H., and Madden, G. O., *Sci. Agr.*, **13**, 194–97 (1932)
15. Berkeley, G. H., and Madden, G. O., *Sci. Agr.*, **13**, 455–57 (1933)
16. Berkeley, G. H., and Willison, R. S., *Proc. Can. Phytopathol. Soc.*, **11**, 21–22 (1944)
17. Berkeley, G. H., and Willison, R. S., *Phytopathology*, **38**, 509–18 (1948)
18. Brase, K. D., and Parker, K. G., *Plant Disease Reptr.*, **39**, 358–62 (1955)
19. Brase, K. D., and Parker, K. G., *Am. Nurseryman*, **104**, 12–13, 70–74 (1956)
20. Breed, R. S., Murray, E. G. D., and Hitchens, A. P., *Bergey's Manual of Determinative Bacteriology*, 6th ed. (The Williams & Wilkins Co., Baltimore, Md., 1529 pp., 1948)
21. Bridgmon, G. H., and Walker, J. C., *Phytopathology*, **42**, 65–70 (1952)
22. Brierley, P., *Plant Disease Reptr.*, **37**, 343–45 (1953)
23. Brierley, P., *Yearbook of Agr. (U.S. Dept. Agr.) Separ. No. 2478*, 596–601 (1953)

24. Brierley, P., *Yearbook of Agr.* (*U.S. Dept. Agr.*) *Separ. No. 2482,* 636–42 (1953)
25. Brierley, P., *Bull. Natl. Chrysanthemum Soc.,* **10,** 47–55 (1954)
26. Brierley, P., *Phytopathology,* **44,** 696–99 (1954)
27. Brierley, P., *Phytopathology,* **47,** 39-43 (1957)
28. Brierley, P., and Smith, F. F., *Phytopathology,* **34,** 506–7 (1944)
29. Brierley, P., Smith, F. F., and McWhorter, F. P., *Yearbook of Agr.* (*U.S. Dept. Agr.*) *Separ. No. 2477,* 608–11 (1953)
30. Broadbent, L., *Investigations of Virus Diseases of Brassica Crops* (Cambridge Univ. Press, Cambridge, England, 94 pp., 1957)
31. Cameron, H. R., and Moore, J. D., *Phytopathology,* **46,** 635 (1956)
32. Cation, D., *Phytopathology,* **31,** 1004–10 (1941)
33. Cation, D., *Phytopathology,* **39,** 37–40 (1949)
34. Cochran, G. W., and Chidester, J. L., *Virology,* **4,** 390–91 (1957)
35. Cochran, L. C., *Science,* **104,** 269–70 (1946)
36. Cochran, L. C., *Phytopathology,* **46,** 635 (1956)
37. Cooley, L. M., *N.Y. State Agr. Expt. Sta. Bull., No. 665* (1936)
38. Cooley, L. M., *N.Y. State Agr. Expt. Sta. Bull., No. 675* (1936)
39. Cooley, L. M., *Phytopathology,* **26,** 44 (1936)
40. Cruz, E. E., *Philippine J. Agr.,* **19,** 209–55 (1956)
41. Darrow, G. M., McGrew, J. R., and Scott, D. H., *U.S. Dept. Agr. Leaflet, No. 414,* 1–8 (1957)
42. Duggar, B. M., and Armstrong, J. K., *Ann. Missouri Botan. Garden,* **10,** 191–212 (1923)
43. Fawcett, H. S., *Citrus Diseases and Their Control,* 2nd ed. (McGraw-Hill Book Co., Inc., New York and London, 656 pp., 1936)
44. Fink, H. C., *Phytopathology,* **40,** 9 (1950)
45. Fink, H. C., *Phytopathology,* **45,** 320–23 (1955)
46. Frazier, N. W., *Plant Disease Reptr.,* **37,** 606–8 (1953)
47. Fridlund, P. R., *Phytopathology,* **44,** 488–89 (1954)
48. Fulton, R. W., *Phytopathology,* **42,** 413–16 (1952)
49. Fulton, R. W., *Phytopathology,* **46,** 12 (1956)
50. Fulton, R. W., *Phytopathology,* **46,** 694 (1956)
51. Fulton, R. W., *Phytopathology,* **47,** 215–20 (1957)
52. Fulton, R. W., *Phytopathology,* **47,** 683–87 (1957)
53. Gardner, F. E., Marth, P. C., and Magness, J. R., *Proc. Am. Soc. Hort. Sci.,* **48,** 195–99 (1946)
54. Giddings, N. J., *J. Agr. Research,* **56,** 883–94 (1938)
55. Giddings, N. J., *J. Agr. Research,* **69,** 149–57 (1944)
56. Giddings, N. J., *Phytopathology,* **36,** 38–52 (1946)
57. Gilmer, R. M., *Plant Disease Reptr.,* **39,** 727–28 (1955)
58. Gilmer, R. M., *Phytopathology,* **46,** 127–28 (1956)
59. Gilmer, R. M., *Phytopathology,* **46,** 241 (1956)
60. Gilmer, R. M., *Plant Disease Reptr.,* **41,** 11–16 (1957)
61. Gilmer, R. M., and Brase, K. D., *Plant Disease Reptr.,* **40,** 767–70 (1956)
62. Gloyer, W. O., and Glasgow, H., *N. Y. State Agr. Expt. Sta. Bull., No. 555,* 1–27 (1928)
63. Gortner, R. A., *Science,* **87,** 529–30 (1938)
64. Harris, R. V., and King, M. E., *J. Pomol. Hort. Sci.,* **19,** 227–42 (1942)

65. Henderson, R. G., *Phytopathology*, **21**, 225–29 (1931)
66. Hildebrand, E. M., *Phytopathology*, **29**, 369–71 (1939)
67. Hildebrand, E. M., *Contribs. Boyce Thompson Inst.*, **11**, 485–96 (1941)
68. Hildebrand, E. M., *Phytopathology*, **31**, 11 (1941)
69. Hildebrand, E. M., *Phytopathology*, **31**, 353–55 (1941)
70. Hildebrand, E. M., *Science*, **95**, 52 (1942)
71. Hildebrand, E. M., *Am. J. Botany*, **29**, 362–66 (1942)
72. Hildebrand, E. M., *Phytopathology*, **32**, 712–19 (1942)
73. Hildebrand, E. M., *Phytopathology*, **32**, 741–51 (1942)
74. Hildebrand, E. M., *Plant Disease Reptr.*, **26**, 454–55 (1942)
75. Hildebrand, E. M., *Am. Nurseryman*, **75**, 9–10 (1942)
76. Hildebrand, E. M., *N. Y. Nursery Notes*, **12**, 6–7 (1942)
77. Hildebrand, E. M., *Phytopathology*, **33**, 6 (1943)
78. Hildebrand, E. M., *Phytopathology*, **33**, 18–19 (1943)
79. Hildebrand, E. M., *Proc. Am. Soc. Hort. Sci.*, **43**, 124–28 (1943)
80. Hildebrand, E. M., *Science*, **100**, 147–48 (1944)
81. Hildebrand, E. M., *Plant Disease Rept.*, **28**, 993–94 (1944)
82. Hildebrand, E. M., *Plant Disease Rept.*, **28**, 1057–60 (1944)
83. Hildebrand, E. M., *Phytopathology*, **35**, 47–50 (1945)
84. Hildebrand, E. M., *Am. Nurseryman*, **82**, 5–8, 18–21 (1945)
85. Hildebrand, E. M., *Plant Disease Reptr.*, **29**, 196–97 (1945)
86. Hildebrand, E. M., *Cornell Univ. Agr. Expt. Sta. Mem., No. 323*, 1–54 (1953)
87. Hildebrand, E. M., *Plant Disease Reptr., Suppl. 222*, 185–223 (1953)
88. Hildebrand, E. M., *Plant Disease Reptr.*, **38**, 572–73 (1954)
89. Hildebrand, E. M., *Science*, **123**, 506–7 (1956)
90. Hildebrand, E. M., *Phytopathology*, **46**, 233–34 (1956)
91. Hildebrand, E. M., *Science*, **126**, 751–53 (1957)
92. Hildebrand, E. M., Berkeley, G. H., and Cation, D., Compilers, *Mich. State Univ. Agr. Expt. Sta., Misc. Publ.*, 1–76 (1942)
93. Hildebrand, E. M., and Curtis, O. F., *Science*, **95**, 390 (1942)
94. Hildebrand, E. M., and Mills, W. D., *Phytopathology*, **31**, 355–56 (1941)
95. Hitchborn, J. H., *Virology*, **3**, 243–44 (1957)
96. Hobart, O. F., and Buchholtz, W. F., *Iowa State Coll. J. Sci.*, **31**, 277–85 (1955)
97. Hobart, O. F., Fink, H. C., and Buchholtz, W. F., *Iowa State Coll. J. Sci.*, **30**, 249–53 (1955)
98. Hockey, J. F., *Sci. Agr.*, **21**, 242–43 (1941)
99. Hockey, J. F., *Sci. Agr.*, **23**, 11 (1943)
100. Holmes, F. O., *Phytopathology*, **24**, 845–73 (1934)
101. Holmes, F. O., *Phytopathology*, **29**, 431–36 (1939)
102. Holmes, F. O., *Handbook of Phytopathogenic Viruses* (Burgess Publishing Co., Minneapolis, Minn., 221 pp., 1939)
103. Holmes, F. O., *Bergey's Manual of Determinative Bacteriology, Suppl. 2, Order Virales*, 6th ed., 1125-84 (The Williams & Wilkins Co., Baltimore, Md., 1529 pp., 1948)
104. Hughes, W. A., and Lister, C. A., *Nature*, **164**, 880 (1949)
105. Iwanowski, D., *Bull. acad. imp. Sci. (Petrograd) Ser. 4*, **3**, 67–70 (1892)
106. Jackson, L. W. R., *Phytopathology*, **23**, 83–90 (1933)
107. Johnson, J., *Wisconsin, Univ., Agr. Expt. Sta. Research Bull.*, 63 (1925)

108. Jones, L. K., Anderson, E. J., and Burnett, G., *Phytopathol. Z.*, **7**, 93–115 (1934)
109. Keitt, G. W., and Clayton, C. N., *Phytopathology*, **29**, 821–22 (1939)
110. Keitt, G. W., and Clayton, C. N., *Phytopathology*, **30**, 13 (1940)
111. Keitt, G. W., and Clayton, C. N., *Wisconsin Horticulturist*, **30**, 276 (1940)
112. Keitt, G. W., and Clayton, C. N., *Phytopathology*, **31**, 14 (1941)
113. Keitt, G. W., and Moore, J. D., *Phytopathology*, **33**, 1213–15 (1943)
114. Keller, J. R., *Florist's Review* (Nov. 2, 1950)
115. Kent, G. C., *The Philippine Agriculturist*, **37**, 555–77 (1954)
116. Keur, J. Y., *Bull. Torrey Botan. Club*, **6**, 53–70 (1934)
117. Keyworth, W. G., *Wallerstein Labs. Communs.*, **8**, 99–109 (1945)
118. Keyworth, W. G., and Davies, D. L. G., *J. Pomol. Hort. Sci.*, **22**, 134–39 (1946)
119. Keyworth, W. G., and Paine, J., *Ann. Rept., East Malling Research Sta., Kent, 1949*, 174–79 (1950)
120. Knorr, L. C., *Phytopathology*, **46**, 557–60 (1956)
121. Koch, K. L., *Phytopathology*, **23**, 317–42 (1933)
122. Kohler, E., *Phytopathol. Z.*, **7**, 1–30 (1934)
123. Kristensen, H. R., *Tidsskr. Planteavl.*, **60**, 69–166 (1956)
124. Kunkel, L. O., *Phytopathology*, **26**, 809–30 (1936)
125. Kunkel, L. O., *Am. J. Botany*, **23**, 683–86 (1936)
126. Kunkel, L. O., *Ann. Rev. Microbiol.*, **1**, 85–100 (1947)
127. Lackey, C. F., *Phytopathology*, **19**, 975–77 (1929)
128. Lackey, C. F., *Phytopathology*, **19**, 1141–42 (1929)
129. Lackey, C. F., *Phytopathology*, **21**, 123–24 (1931)
130. Lackey, C. F., *J. Agr. Research*, **44**, 755–65 (1932)
131. Lackey, C. F., *J. Agr. Research*, **55**, 453–60 (1937)
132. Lesley, J. W., and Wallace, J. M., *Phytopathology*, **28**, 548–53 (1938)
133. Luria, S. E., *General Virology* (John Wiley & Sons, Inc., New York, N.Y., 427 pp., 1953)
134. Mackinnon, J. P., *Proc. Can. Phytopathol. Soc.*, **24**, 20 (1956)
135. MacClement, W. D., and Richards, M. G., *Can. J. Botany*, **34**, 793–99 (1956)
136. McKinney, H. H., *Plant Disease Reptr.*, **40**, 1102–5 (1956)
137. McKinney, H. H., *Plant Disease Reptr.*, **41**, 254–55 (1957)
138. McKinney, H. H., and Clayton, E. E., *Phytopathology*, **34**, 60–76 (1944)
139. McKinney, H. H., Paden, W. R., and Koehler, B., *Plant Disease Reptr.*, **41**, 256–66 (1957)
140. McWhorter, F. P., and Brierley, P., *North Am. Lily Soc.*, 1955, 10–27 (1955)
141. Manns, T. F., and Manns, M. M., *Trans. Peninsula Hort. Soc.*, **48**, 72–76 (1935)
142. Mayer, A., *Landwirtsch. Vers.-Sta.*, **32**, 451–67 (1886)
143. Milbrath, J. A., *Phytopathology*, **46**, 638–39 (1956)
144. Milbrath, J. A., *Phytopathology*, **47**, 655–47 (1957)
145. Millikan, D. F., and Guengerich, H. W., *Phytopathology*, **46**, 130 (1956)
146. Mills, W. D., *Phytopathology*, **36**, 353–58 (1946)
147. Moore, J. D., Boyle, J. S., and Keitt, G. W., *Science*, **108**, 623–24 (1948)
148. Moore, J. D., and Keitt, G. W., *Phytopathology*, **39**, 15–16 (1949)
149. Mulder, D., *Tijdschr. Plantenziekten*, **60**, 265–66 (1954)
150. Murphy, P. A., *Nature*, **138**, 955 (1936)

151. Nelson, R., *Mich. State Univ. Agr. Expt. Sta. Techn. Bull.*, **58**, 1–28 (1922)
152. Oberholzer, P. C. J., Mathews, I., and Stiemie, S. F., *Union S. Africa Dept. Agr. Sci. Pamph.*, No. 297, 1–18 (1949)
153. Ocfemia, G. O., *Philippine Agriculturist*, **33**, 142–44 (1949)
154. Olson, E. O., and McDonald, J. R., *Plant Disease Reptr.*, **38**, 439–41 (1954)
155. Olson, E. O., and Sleeth, B., *Proc. Ann. Rio Grande Valley Hort. Inst., 8th Inst.*, 84–88 (1954)
156. Palmiter, D. H., and Parker, K. G., *Phytopathology*, **38**, 20 (1948)
157. Parker, K. G., and Palmiter, D. H., *Plant Disease Reptr.*, **32**, 188–90 (1948)
158. Plakidas, A. G., *Plant Disease Reptr.*, **39**, 525–41 (1955)
159. Posnette, A. F., and Cropley, R., *J. Hort. Sci.*, **31**, 298–302 (1956)
160. Posnette, A. F., and Ellenberger, C. E., *Ann. Appl. Biol.* **45**, 74–80 (1957)
161. Price, W. C., *Contribs. Boyce Thompson Inst.*, **4**, 359–403 (1932)
162. Price, W. C., *Phytopathology*, **26**, 665–75 (1936)
163. Price, W. C., *Am. J. Botany*, **25**, 603–12 (1938)
164. Price, W. C., *Am. Naturalist*, **74**, 117–28 (1940)
165. Price, W. C., *Quart. Rev. Biol.*, **15**, 338–61 (1940)
166. Raleigh, W. P., *Phytopathology*, **26**, 795 (1936)
167. Rankin, H. W., *N. Y. State Agr. Expt. Sta. Techn. Bull.*, 175 (1931)
168. Rawlins, T. E., and Parker, K. G., *Phytopathology*, **21**, 331–35 (1931)
169. Reeves, E. L., and Cheney, P. W., *Phytopathology*, **46**, 639 (1956)
170. Reeves, E. L., and Cheney, P. W., *Phytopathology*, **46**, 639–40 (1956)
171. Richards, B. L., *Utah Acad. Sci. Proc.*, **22**, 9 (1945)
172. Richards, B. L., Hutchins, L. M., and Reeves, E. L., *Phytopathology*, **39**, 19 (1949)
173. Richards, B. L., Reeves, E. L., and Hutchins, L. M., *Phytopathology*, **36**, 409 (1946)
174. Schultz, E. S., and Folsom, D., *J. Agr. Research*, **25**, 43–48 (1923)
175. Schultz, E. S., and Folsom, D., *J. Agr. Research*, **30**, 493–528 (1925)
176. Smith, K. M., *Ann. Appl. Biol.*, **16**, 209–29 (1929)
177. Smith, K. M., *Proc. Roy. Soc.* (*London*), [B]**109**, 251–67 (1931)
178. Smith, K. M., *Biol. Revs. Biol. Proc. Cambridge Phil. Soc.*, **8**, 136–79 (1933)
179. Smith, K. M., *Parasitology*, **29**, 70–85 (1937)
180. Smith, K. M., *Textbook of Plant Virus Diseases* (J. & A. Churchill, Ltd., London, Engl., 615 pp., 1937)
181. Smith, K. M., *Textbook of Plant Virus Diseases*, 2nd ed. (Little, Brown & Co., Boston, Mass., 652 pp., 1957)
182. Smith, K. M., and Bald, J. G., *Parasitology*, **27**, 231–45 (1935)
183. Stanley, W. M., *Phytopathology*, **26**, 305–20 (1936)
184. Stewart, F. C., *N. Y. State Agr. Expt. Sta. Bull., No. 463*, 157–88 (1919)
185. Thomas, H. E., *Hilgardia*, **10**, 581–88 (1937)
186. Thomas, H. E., *Phytopathology*, **32**, 435–36 (1942)
187. Thomas, H. E., and Hildebrand, E. M., *Phytopathology*, **26**, 1145–48 (1936)
188. Thomas, H. E., and Scott, C. E., *Phytopathology*, **43**, 218–19 (1953)
189. Thomas, H. E., Scott, C. E., Wilson, E. E., and Freitag, J. H., *Phytopathology*, **34**, 658–61 (1944)
190. Travis, R. V., and Brierley, P., *Plant Disease Reptr.*, **41**, 524 (1957)
191. Valleau, W. D., *Phytopathology*, **25**, 37 (1935)
192. Vanterpool, T. C., *Phytopathology*, **16**, 311–31 (1926)

193. Vaughan, E. K., *Tijdschr. Plantenziekten*, **62**, 271–73 (1956)
194. Wagnon, H. K., and Breece, J. R., *Calif. Dept. Agr. Bull.*, **45**, 256–58 (1956)
195. Wallace, J. M., *Phytopathology*, **29**, 743–49 (1939)
196. Wallace, J. M., *Phytopathology*, **30**, 673–79 (1940)
197. Wallace, J. M., *J. Agr. Research*, **69**, 187–214 (1944)
198. Wallace, J. M., in *Viruses, 1950* (Delbrück, M., Ed., California Institute of Technology, Pasadena, Calif., 1950)
199. Wallace, J. M., and Drake, R. J., *Citrus Leaves*, **35**, 8–9, 23 (1955)
200. Wallace, J. M., Oberholzer, P. C. J., and Hofmeyer, J. D. J., *Plant Disease Reptr.*, **40**, 3–10 (1956)
201. Waters, C. E., *Plant World*, **1**, 83–84 (1898)
202. Willison, R. S., *Canadian Disease Survey, 28th Ann. Rept. (1948)*, 79 (1949)
203. Willison, R. S., Berkeley, G. H., and Chamberlin, G. C., *Phytopathology*, **38**, 776–92 (1948)
204. Wingard, S. A., *J. Agr. Research*, **37**, 127–53 (1928)

# PLANT TISSUE CULTURES OPEN A BOTANICAL FRONTIER[1,2]

By A. J. Riker and A. C. Hildebrandt[3]
*Department of Plant Pathology, University of Wisconsin, Madison, Wisconsin*

## Introduction

The technique of growing tissues from higher plants on media in glass containers has opened the way for numerous fundamental and applied investigations. Thus the procedures used so successfully with numerous microorganisms can be applied to higher plant tissues. This valuable technique has advanced beyond the point where it is an end in itself. Now it permits the extension of interesting problems already encountered in the botanical field into important new pathways.

The early history and much literature about plant tissue culture have already been covered in various reviews or conferences, notably those reported by Gautheret (27); Klein (47); Skoog & Miller (88); Steward & Shantz (92); and White (101 to 104). The work with plant tissue cultures has derived many ideas and techniques from that done with animal tissue cultures, where the amount of research has been far more extensive [e.g., Parker (76, 77); Murray (68)]. However, the plant tissues make unlimited active growth on media containing only nutrients with known chemical formulae. Furthermore, many plant tissues grow well for a month or more without a change in nutrient. Frequently, the amount of growth is determined by weighing the tissue. Plant tissues have great usefulness in fundamental studies of growth.

In the present discussion, no comprehensive review was attempted. Instead, some of the pathways in this new frontier have been selected for discussion, including their significance and the areas opened by them. In this way, the writers hope that beginners and those unfamiliar with the field will find new views of special interest. Many details in the older literature as well as considerations of root culture, embryo culture, endosperm culture, stem tip culture, and temporary cultures of leaves and sections have been almost entirely omitted because of the limitations of space.

[1] The survey of the literature pertaining to this review was concluded in January, 1958.

[2] The following abbreviations are used: DNA (deoxyribonucleic acid); IAA (indole-3-acetic acid); RNA (ribonucleic acid); TMV (tobacco mosaic virus).

[3] The writers are indebted to a number of fellow investigators for viewpoints and ideas, especially those concerning the significance of the results secured and of the important new areas opened for development. These viewpoints have been given freely not only in publications but also in correspondence as well as in public and private discussions.

### Technique for Isolation

*Normal tissue.*—The isolation of normal tissue is dependent upon the stimulation of the callus type of growth. One of the simpler techniques is the following: a normal stem of sunflower not far from the growing tip is selected. The epidermis is removed aseptically. A cylinder about 10 mm. long is placed upright on a suitable medium [e.g., Hildebrandt, Riker & Duggar (38)] in an Erlenmeyer flask, prescription bottle, or large test tube. The medium basically contains nitrate for nitrogen, sucrose for carbon, balanced mineral salts, and a trace of α-naphthaleneacetic acid. This encourages growth, inhibits the formation of roots from the cylinder, and favors the development of callus. Simple variations of this technique include aseptic removal of portions of other actively growing tissues. Ordinarily, the size needs to be at least several millimeters in diameter. For certain difficult normal tissues, complex substances, notably yeast extract and coconut milk, are added. The medium usually contains 0.5 to 0.7 per cent agar. This is just solid enough to support the tissue and fluid enough for a ready exchange of nutrient elements and to prevent desiccation for some time.

The synthetic medium first used by White and certain improvements in it are given by Reinert & White (80). Here also are briefly reviewed almost a dozen publications by White and his associates, which have been so important in the development of tissue culture studies.

For incubation, a temperature between 25° and 28°C. and an air humidity of about 55 per cent for a month have proved satisfactory. When tissue transplants are made after 4 to 6 weeks, the portion of tissue used should ordinarily weigh more than 25 mg. or it may fail to grow consistently.

*Tumor tissue.*—For tissue from galls on tobacco hybrids or from legumes with wound tumor virus, the technique is like that used for normal tissue. The technique of isolating tissues from crown gall provides some added difficulties. The best experimental material comes from the secondary galls [White & Braun (105)], some of which may be free from the inciting crown gall bacteria. To secure such secondary galls, puncture inoculations are made close behind the condensed apical bud of plants like sunflower and Paris-daisy. In some cases, with rapidly-growing plants, the apical bud expands, spreading the bacteria from the original inoculation out over a number of internodes and along certain petioles.

The galls which grow sufficiently to break the epidermis either of the stem or the leaf petiole on which they occur usually contain bacteria. For tissue cultures, secondary galls which have not broken through the epidermis are selected and the tissue mass removed aseptically to an agar medium for incubation. While one may be fortunate the first time, usually a number of trails are necessary to secure uncontaminated tissue which grows well.

An alternate procedure is to isolate tissue from a rapidly growing crown gall. The galls used should be reasonably free from surface contamination. Since the causal bacteria occur in isolated pockets throughout the gall, one

has to secure tissue that is free of these pockets. Thus, one may slice away the contaminated surface. With a sterile knife for each cut, a number of pieces of gall tissue about 2 mm. across are transferred to agar. Among one hundred such pieces perhaps 25 will be free of microorganisms and half a dozen will make some growth. Of these, perhaps two or three will grow vigorously.

Cultures secured in this manner sometimes contain microorganisms which do not show as contaminants with nitrate-sucrose-mineral salts media. An important routine in any tissue culture work is to macerate representative portions of the tissue being studied, to make dilution plates with a rich medium containing perhaps yeast extract, coconut milk, peptone, or similar rich sources of nutrients as described by Robbins & Hervey (84). Likewise, some cultures have been found to contain fungi which fail to appear until the culture is partly dried out.

The terminology commonly applied to these cultures may not be too precise. "Normal" cultures are those derived from normal tissue and "tumor" or "diseased" cultures are those derived from "tumor" tissue. Although the term "normal" is used, one realizes that it may be different from the tissue in the intact plant from which it was derived.

## Physical Conditions for Culture

With the possibility of growing tissues from higher plants in culture as many microorganisms are grown, it became necessary to determine the most favorable conditions for growth. This included experiments both on physical and on chemical conditions. Obviously, the metabolic studies would be considerably enhanced if physical and nutritive circumstances could be developed which would define exactly the circumstances for incubation and if one knew the chemical formula for every nutrient supplied.

The temperature range for growth was found to be approximately the same for the tissues in culture as it was for the whole plant. For many tissues, growth was satisfactory between 20° and 28°C. and was optimum at approximately 26° to 27°C. Growth was reduced at temperatures much lower or much higher than this range.

The amount of existing moisture presented certain complex situations. When the tissues were grown on agar, the best results commonly were secured when the agar was quite dilute, approximately 0.5 to 0.7 per cent. This provided support for the tissue and good contact with the agar base with, simultaneously, excellent aeration from the atmosphere. Furthermore, the agar in such a liquid state held its moisture over relatively long incubation periods and made frequent transfers of cultures to new media unnecessary. The incubation periods of one to two months or sometimes longer were entirely feasible. The relative humidity in the air of the incubation room was most favorable at about 55 per cent. Humidities approaching saturation were undesirable because molds sometimes grew through the cotton plugs, and because the tissues grew less well. Possibly the nutrients

from the agar moved into the tissue partly because of some evaporation from the surface of the tissue. The osmotic pressure was, no doubt, a factor in the best balance in the concentration of mineral salts.

The hydrogen-ion concentration was examined [Hildebrandt, Riker & Duggar (37)] over the usual range. During the long incubation period, the pH of media on the acid side tended to drift toward neutrality, while that on the alkaline side also tended to drift toward neutrality. In part, the drift from the acid side came from the utilization of nitrate from sodium, potassium, or calcium nitrate in the nutrient solution. From the alkaline direction toward neutrality the drift, at least partly, was the result of atmospheric and metabolic $CO_2$ which formed carbonic acid with the water in the medium. In general, the most favorable pH for the growth of tissues was approximately 6.0 or that found in nature in the host plants.

The oxygen tension in the medium is important since it appears to be a limiting factor for growing tissues in liquid cultures. White (100) observed the formation of a few leaves on submerged and slowly growing tobacco tissues. He noted the lack of oxygen in association with the morphological changes. In studies of critical means for aerating the cultures, Muir, Hildebrandt & Riker (67) have shown that rather strong agitation of the liquid medium has encouraged the best growth. Steward and his associates have grown their cultures in rotating tubes so the tissues were exposed to atmospheric oxygen approximately two-thirds of the time. This effort to provide excellent growth in liquid medium has been stimulated also by efforts to analyze the metabolic products that are present in the medium after the tissues have incubated a suitable time. Such analyses in the presence of agar are confronted with a number of difficulties.

The ability of plant tissues to grow in a synthetic medium has necessitated a suitable balance among the mineral salts. Such balances have provided a number of different media which resemble one another rather closely, but which vary in different constituents, depending upon the tissue being studied. The formulae for several of these media are given by White (101), Hildebrandt, Riker & Duggar (38), and others. Skoog (87) has pointed out that plant tissue cultures are not so well suited as other materials for the study of trace elements.

### Modified Morphology and Physiology of Normal Tissue

The importance of auxins in the establishment of cultures from normal carrot tissues was demonstrated by Gautheret and extended by various investigators, especially those in his laboratory [Gautheret (27)]. He considered that auxin was not only a simple growth substance but also a cell division substance. The comparison of normal and crown gall tissue in culture had shown that, while crown gall tissue did not require added auxin, it was the only additional substance necessary for the growth of certain normal tissues. Kulescha & Gautheret (52) and Kulescha (51) have demonstrated that tumor tissue elaborated more auxin than normal tissues.

These results have been the point of departure for numerous researchers on growth stimulation and on tumor formation. Bitancourt (6) has identified the growth substances as predominantly indole-3-acetic acid, with some of its ethyl ester and indoleacetonitrile. In normal and tumor tissue, the auxins appeared identical.

The phenomenon of "anergie," described by Gautheret (29) and by various others, is of special interest. While important in itself, it opens the way for studies of variations in relation to other critical characteristics. Certain normal tissues, unable to grow without added auxin, after a time developed the ability to grow without this supplement. Apparently, tissue showing this same phenomenon has been called "habituated" or "accustomed." It was peculiar to the variability of the physiological properties of tissue cultures and was not manifest in the entire normal plant. Such "anergised" tissues produced tumors when grafted back into normal tissue. The ideas about tumor formation should take into consideration this kind of transformation which took place under strictly aseptic conditions. Furthermore, such cultures were advantageous for studies of the factors influencing differentiation and dedifferentiation. These and a great many related investigations have been reviewed by Gautheret (28) and have been reported at the Briançon meetings [Gautheret (27)]. Since the large number of papers by Gautheret and his associates have been so well reviewed recently, only an outline of their content and significance has been attempted here.

To make comparisons between normal and diseased tissue in culture, Henderson, Durrell & Bonner (32) developed a medium for normal sunflower tissue which contained supplements of vitamins, amino acids, adenine, and auxin. The healthy tissue grew slowly but continuously. An habituated form appeared. Whereas normal and diseased tissues were capable of approximately equal production of auxin [Henderson & Bonner (31)], the normal tissue appeared to possess one or more inhibiting factors which prevented the maintenance of a level of auxin required for normal growth. With prolonged heating of tumor tissue at 33° to 34°C. for 3 to 6 weeks, some critical factor for growth was destroyed [Rier & Henderson (81)]. At 25°C., it regained its growth rate only if it were given supplements of the vitamins, amino acids, adenine, and auxin mentioned above. They considered that the ability to provide such growth substances was characteristic of the tumor tissue and not of the normal tissue.

A beginning has been made by Gautheret, Camus, and others in Gautheret's laboratory to describe the developmental anatomy of massive plant tissue cultures. So far this has been largely descriptive, but points the way toward experimental control of the developmental processes. This was reviewed, e.g., by Gautheret (27, 28, 30).

The technique of tissue culture was extended by Morel to a great number of plants in different genera, including *Vitis, Parthenocisus, Rubus, Nicotiana,* and *Malva* [Morel (61)]. Only a few of his more than 40 papers can

be reviewed here. With coconut milk, Morel (62) and Morel & Wetmore (65) grew some monocotyledons, ferns, and *Selaginella*. When Bove & Morel (9) started with very young tissues from the procambium in the apex or from the embryo, they extended the range of species, e.g., to citrus and lupinus. As Morel has said in discussions, these parenchymas growing in a perfectly defined medium are as easy to handle as microorganisms. They have none of the complications arising from differentiation into organs. They are ideal for the study of metabolism at the cell level, especially with labelled compounds.

With higher plants, the growth of excised meristems was abnormal and very slow. Morel (63) tried additions to the medium of B vitamins, mixtures of amino acids, SH compounds, adenine, and kinetin, which improved growth. Finally, gibberellin gave normal growth. It seemed to have the qualifications for Went's caulocaline. This technique has been valuable for freeing crop plants from viruses as will be discussed later.

From these numerous studies, Morel and his associates found that auxin was necessary for cell division in many tissues of higher plants, but lower plants did not require added auxin. The habituation to auxin was shown to be general. These tissue cultures provide a tool for many physiological studies, such as auxin metabolism in the cell, and transformations of the common metabolic substances such as amino acids and organic acids.

Various growth stimulating substances have been examined by Steward and his associates. The results have been reported in over 40 publications. They are reviewed by Steward & Shantz (92), so no attempt has been made here more than to outline something of the breadth and direction of their researches. An ingenious procedure was developed by Caplin & Steward (21) in which cross sections of carrot root weighing about 4 mg. were cut aseptically from the phloem just outside the cambium with a canula. They were grown in a liquid medium on a rotating drum. The tissue was alternately exposed to the air and bathed in the liquid; it grew with little variability. Determinations of fresh and dry weight, cell count, and protein synthesis served for measurements of growth. Caplin (20) discussed the use of weight as a measure of growth. The size of the original transplant and the incubation period were important. In many cases, the total amount of growth was large either because of the size of the initial transplant or because of the length of the incubation period, while the relative amount of growth was small. Cells having various sizes, shapes, and degrees of differentiation were observed in the cultures.

Natural growth stimulating substances from fluid endosperms (e.g., from coconut milk, aesculus, and maize) were fractionated. The isolated active fractions or isolated single substances rarely reproduced the full growth effects of whole extracts. The effects from natural materials were due to complex mixtures. Various apparently unrelated substances, such as diphenylurea, and Fractions C and D from coconut milk, leucoanthocyanins and chlorogenic acids from aesculus, as well as indole-3-acetic acid (IAA),

glucoside, and purines from maize, were partially active. Apparently, as differentiation proceeded, factors for random proliferation were regulated by substances or mechanisms. To determine what they may be is not only of interest but imperative [Steward & Caplin (89); Steward & Shantz (91)].

The changes in morphology and structure of cultures from carrot and rose were observed during 18 years by Nobecourt (74). The cultures grew both with and without auxin. Vigorous callus growths were observed; they were both green and white, with and without lignified cells. In some cases, roots developed and in others tiny shoots and leaves. Earlier details were cited in ten previous papers.

The kind and degree of growth response have appeared to depend on various interactions. This has had different aspects—for example, the interactions of different chemical substances which occurred in several mixtures in nature. Among the nitrogenous compounds were casein hydrolysates, ammonium salts, nitrate salts, and urea. The results with substituted 6-aminopurines and indole-3-acetic acid were of special interest. Various aspects of the nitrogen metabolism have been reviewed by Steward & Pollard (90). Other interactions were dependent on inherent variables within tissues from the same source. With carrot root pieces, there were the variations to pure compounds as diphenylurea, to various fractions from coconut milk, to nitrogen supplements as casein hydrolysate, and to indole-3-acetic acid. However, tissues from different species did not always react alike. Carrot tissue responded to coconut milk and other natural extracts. Artichoke tissue responded to benzothiazolyloxyacetic acid. Potato responded to mixtures of natural endosperm and chemical growth regulators. From work of this kind, it seems evident that there exists in the plant kingdom a range of substances specifically active in stimulating cell division in the mature parenchyma of carrot roots and of certain other cells, such as artichoke tuber and potato tuber [Steward & Shantz (91)].

Chemical regulation of growth and organ formation has been achieved by Skoog and his associates [reviewed, Skoog & Miller (88)]. The tissue culture technique with tobacco pith callus permitted a closer examination of individual factors and of their interactions in various growth processes than has been possible so far with intact plants. A delicate quantitative balance between indole-3-acetic acid and adenine and between these and other factors determined the type of growth and organ formation that occurred [Skoog & Tsui (88a, 88b); Miller & Skoog (58)]. A cell division factor was found in vascular tissue and various natural products.

A crystalline substance, isolated and identified as 6-(furfurylamino) purine, was synthesized [Miller *et al.* (59)] and was named kinetin because of its activity in promoting cell division. A number of analogues, mainly adenine derivatives, possessed similar activity to varying degrees and were called kinins [Okumura *et al.* (75)].

The quantitative effects of IAA and kinetin were considered primarily

growth regulating actions. But, the morphogenic influences were numerous and subject to modification by various factors and conditions, including high phosphate levels. With tobacco callus or stem-tissue cultures on White's medium and with 2 mg./l. level of IAA, the amount of kinetin was varied [Miller & Skoog (58)]. Cell enlargement and a few short roots occurred in the controls. The effect of kinetin at 0.02 mg./l. was to increase undifferentiated callus growth; but at 0.5 and 1.0 mg./l. it was mainly on bud formation and the repression of roots. Still more inhibited growth but was not very toxic. Comparable results appeared when the amounts of IAA were varied and kinetin kept constant at 0.2 or at 1.0 mg./l. This ability to influence the production of callus, roots, or shoots by varied concentrations of auxin and kinetin has been a striking and important contribution to morphogenesis.

The use of triiodobenzoic acid, which influenced auxin transport to bring about the formation of buds and possibly flower buds, was further critical evidence about morphogenesis [Niedergang-Kamien & Skoog (71)]. The stimulating effect of casein hydrolysate appeared in the presence of kinetin and IAA. This benefit was explained largely, if not entirely, by the effect of tyrosine. Tryptophan had the effect of added IAA. Differences in the responses of tissues derived from phloem, cambium, or pith of the same tobacco stem were probably the result of differences in capacities acquired during subculturing. They gave comparable responses in root and bud formation with suitable variations in kinetin and IAA.

The evidence pointed to a uniformity in growth factor requirements and regulatory mechanisms for all types of growth [Skoog & Miller (88)]. Interactions between IAA and kinetin and between these and other factors exerted decisive influences. The general conclusions were opposed to concepts of specific organ-forming substances, minimizing the significance of slight structural modifications. They often must have reflected only slight differences in growth factor contents. Since attempts have failed to secure a single specific root, stem, leaf, and flower-forming substance, evidence has accumulated against its existence. The results also conflicted with the concept of "determination," i.e., irreversible loss in regenerative capacities of cells and tissues in ontogeny.

Furthermore, the results suggested the futility of attempts to interpret growth phenomena on the basis of very precise computations of the interactions between only two factors in a multiple factor system [Skoog & Miller (88)]. Doubtless, kinetin and IAA have been involved in nucleic acid synthesis and metabolism. One could visualize an essentially nuclear mechanism of growth regulation which possessed recognized means for interaction with the cytoplasm. The sharp distinctions between stimuli, "energy-furnishing" metabolites, and structural units have been disappearing, as these have graded into one another. For the understanding of the detailed function or biological action of any one substance, we must learn a great deal about many others.

Neither marigold nor tobacco tissue cultures [Hildebrandt, Riker &

Muir (41)] grew on DNA, but tobacco tissue grew well with RNA as a source of nitrogen. No growth of consequence developed with either tissue on the purines or pyrimidines employed.

After the judicious use of sugar and auxin in a cut made in a lilac callus, strands appeared [Wetmore (98)] in the callus with both xylem and phloem or xylem alone, according to the concentrations employed. More startling was the recognition that these strands tended to form a circle, as they do in stems, with the phloem on the outside and xylem inside. Many details of growth and differentiation from organ cultures in a long series of papers have been reviewed by Wetmore (98).

At a given auxin concentration, potassium, nitrate, and carbohydrates influenced the increase in fresh weight of Jerusalem artichoke tuber tissue in culture [Nitsch & Nitsch (72)]. Indole-3-acetic acid and its ethyl ester were about equally active, while the amide was active at 300 to 1000 times higher. *Cis*-cinnamic acid was active at 1000 times higher.

Organic nitrogenous compounds influenced the growth of Jerusalem artichoke tissue in various ways [Nitsch & Nitsch (73)]. Sterilization by filtration and the use of both D- and L-forms were important. Some D-forms were inhibitory. Glycine, asparagine, allantoin, arginine, alanine, gamma amino butyric, glutamic and aspartic acids, glutamine, or urea could support growth, but the first four were not equal to nitrate. Many other amino acids and related substances were inhibitory.

Tracheids and shoot apices developed in Sequoia cultures only in cells free from tannin [Ball (2)]. The sugar metabolism of cultures from Sequoia was studied by Ball (5) who was the first to secure callus cultures from a gymnosperm. The three normal sugars were sucrose, glucose, and fructose. The other two were manufactured from any one. The callus grew well on mannose, galactose, and raffinose. On galactose, it formed raffinose and on raffinose it formed galactose. *Vinca* crown gall tissue acted similarly.

Isotopes $P^{32}$ and $S^{35}$ were localized principally in the marginal meristems and in the cambia around the tracheid groups of Sequoia cultures [Ball (3)]. $C^{14}$ distribution was similar except it was more general, particularly in the cell walls. $C^{14}$ was long retained in the callus [Ball, (4)]. During subsequent transfers at 2-month intervals, it was transferred to small groups of cells, presumably the precursors of new tracheid groups.

### Tumor Tissue

The investigation with diseased tissues has had as its basis prior experiments carried out on intact plants, particularly those with the crown gall disease. White & Braun (105) with tissue in culture demonstrated the independent nature of the crown gall tumor cell and that its continuous growth was not dependent upon bacterial stimulation [Braun & White (18)]. As a result of the bacterial action, he found that a profound and heritable change had occurred from a normal to a tumor cell. Not only did tumor tissue grow indefinitely in culture, but when small fragments were grafted back into the original plant, they developed typical crown gall without bacteria.

Normal tissues, when grafted, fused with the host and no tumorous growths developed. Thus, the concept of autonomy was placed on a sound basis, and has been repeatedly confirmed.

Cultures made from both normal and tumor tissues of white spruce [Reinert & White (80)] grew on a synthetic medium which was improved over White's basic medium. The rapid blackening and inhibited growth from active polyphenol oxidase were overcome temporarily by ascorbic acid and diethyldithiocarbamate, but continued growth was best with tyrosine. The tumor tissue required vitamin $B_{12}$, which was not required by normal spruce tissue.

The transition from a normal cell to an autonomous crown gall tumor cell was shown [Braun (11, 12, 13)] to be a gradual but progressive process, requiring three or four days of bacterial action. This confirmed work done on intact plants, and was based on the finding that the inciting bacteria in *Vinca* could be destroyed by heat without killing the host cells. The gradual nature of the process was demonstrated by results at different time intervals [Braun (15)]. After only 36 hr. of bacterial action, the slightly altered plant cells grew very slowly both in the host and in tissue culture. After 50 hr., the changing cells grew at a moderately fast rate. After 96 hrs., the completely altered cells grew rapidly both in the host and in tissue culture.

The results suggested a series of well-defined growth substance-synthesizing systems, concerned with growth by cell division, which became gradually but permanently active—systems which were precisely regulated in the normal cells. The fully altered tumor cell was capable of making all the growth factors needed from the nitrate-sucrose-mineral salts medium. Tumor cells which were only partly changed had their requirements only partly satisfied for several essential but well-defined growth factors.

As the tumor cell became more autonomous, its requirements became less exacting. The series of growth substance-synthesizing systems became gradually unblocked during the transition from the normal to the tumor cell. The degree of activation of these systems determined the rate of growth. The difference between rapid and slow growing tumor cells was quantitative. Normal cells did not grow at all on the basic medium. These normal cells had an absolute requirement for auxin and for a factor such as kinetin. A fundamental difference between tumor and normal cell appeared [Braun (14)] thus: the tumor cells could synthesize these essential growth factors, the normal cells could not. These growth substances have induced in normal cells certain morphological and cytological patterns [Braun & Stonier (17)].

Another approach was through cultures from the distinct type of crown gall induced by attenuated bacteria. The attenuation was a gradual process [Van Lanen, Baldwin & Riker (95)]. Such galls were relatively small and were composed of an assortment of abnormal tissues and organs that showed varying degrees of organizations [Locke, Riker & Duggar (57)]. In tissue culture [Braun (13)], these pluripotent plant cells, despite their alteration, retained a highly developed regenerative capacity to produce abnormal leaves and shoots. Such tissue was well-suited to study the recovery of crown gall

tumor cells. Braun felt that crown gall did not arise as a somatic mutation. The factor which caused crown gall tumor cells to develop abnormally was an autonomous or semiautonomous entity subject to the effects of dilution in very rapidly dividing tumor cells.

Differences [Braun (16)] between the tumor cells in tissue culture which developed after contact with the causal bacteria for 96, 50, and 36 hr., were studied further. Growth from the first was excellent, from the second, fair, from the third, poor, and from normal tissue there was none. The first grew well on the nitrate-sucrose-mineral salts medium without supplement. The second, to grow as well as the first, required auxin, meso-inositol and glutamine or a related substance. The third required those substances necessary for the second plus proline and histidine. Normal tissue, required all these materials plus cytodylic acid, guanilic acid, kinetin, and possibly other amino acids. Thus, Braun has demonstrated a striking sequence of metabolic steps between normal and tumor tissue.

Klein (48) has reported the activation of a series of metabolic systems in various stages of crown gall development on carrot disks. Among the critical chemicals for three different stages were (*a*) amino acids and purines; (*b*) kinetin, gibberellic acid, mevalonic acid, and coconut milk; and (*c*) auxin.

Cultures of tumor tissue have provided a good means for checking the balance of many critical factors. In 1942, Riker wrote (82),

> While we shall continue to analyze individual factors that by their presence or absence may change normal into pathological growth, there is another possibility that deserves consideration. This is that in normal growth a number of factors may operate in a suitable balance. However, in pathological growth of one kind, a group of these factors may be out of balance. Likewise, in pathological growth of another kind, the balance is disturbed in another way.

The possibilities for work with suitable balances of important nutritive and metabolic substance called for an understanding of the critical amounts for encouraging or of inhibiting growth. Such amounts can be worked out with tissue cultures in a way not yet possible with whole seed plants. The work often has been limited to tumor tissues in order to cover a broad area. After the critical points were determined, comparisons could be made then with normal tissue.

The importance of graded series appeared in the early studies by Hildebrandt, Riker & Duggar (37). The best growth by sunflower tumor tissue was at 20° to 32°C., pH 5.5 to 6.5, and 1 gm./l of sucrose. With various plant extracts, optima for growth with varying amounts were sometimes striking. With some extracts, growth was encouraged to a specified point and then inhibited [Hildebrandt, Riker & Duggar (39)]. Various extracts gave unlike results; and different tissues behaved in dissimilar ways. In order to clarify the factors favoring growth, a series of studies was made of the simpler nutrients which might stimulate growth. Still more important was the determination of which might be growth inhibitors; as such they would be important factors in an imbalance which would discourage

development of tumor tissue. Moreover, they might influence the resistance of the tissue to pathogenic microorganisms. These studies usually were made on agar where the diffusion rates of entry by nutrients and of exit by metabolic products would be relatively slow. Perhaps these rates were more nearly comparable to those in a plant than in liquid media.

The mineral salts were studied along with glycine, thiamine, pyridoxine, and nicotinic acid in a series of triangular arrangements with sunflower and tobacco tumor tissue. A total of 108 different combinations was employed [Hildebrandt, Riker & Duggar (38)]. The variations were so recorded as to facilitate observation of the growth increase or decrease which appeared with changes in salt or vitamin content. While particularly valuable for the preparation of synthetic media, these results indicated that the influence of plant extracts probably resulted from something other than the balance of mineral salts. Where they have been tried, liquid cultures preferred different and usually more dilute amounts than cultures on 0.6 per cent agar.

All seven of the vitamins studied by Czosnowski (23) were synthesized by carrot tissue. He obtained similar results with *Vitis* and *Parthenocissus*. However, after ten successive transfers on a thiamine-free medium, the chemical tumor tissue of *Vitis* began to grow slowly and to degenerate. Growth was restored with 1 mg./l. of thiamine. This occurred only with the chemical gall. Except for folic acid, the amounts of vitamins in the tumor were less than in the normal tissue—but the reduced amount was never a limiting factor.

Physiological comparisons of grape tissue cultures from normal stems, crown gall, and chemical galls [Czosnowski (24)] revealed some important differences. The crown gall tissue was intermediate between the other two. The chemical tumor (from auxin) was distinguished by maximum activity, but had the lowest respiration activity. The chemical tumor lacked physiological harmony in the isodiametric growth and the separation of the cells. The tumefaction did not depend in both cases upon the same changes in the nature of a normal tissue.

Various growth substances were employed with sunflower and tobacco tumor tissue and commonly stimulated some growth at high dilutions [Hildebrandt & Riker (34)]. However, they usually inhibited growth if stronger than about $10^{-5}$ gm./l. The tissues which grew most rapidly frequently contained the largest number of scalariform cells [Struckmeyer, Hildebrandt & Riker (93)].

The importance of auxin was studied further by Klein & Vogel (49). They reduced the level of indole-3-acetic acid in sunflower tumor tissue cultures with x-rays and a specific antiauxin, thus, inhibiting growth. The inhibition was reversed by appropriate amounts of indole-3-acetic acid. Preliminary results on energy metabolism [Klein (48)] showed that tumor tissues had some unusual and unexplained deviations from the expected metabolic and growth interrelations.

Different simple sources of nitrogen, at graded concentrations, were studied with sunflower gall tissue by Riker & Gutsche (83). Nitrate at .004

to .032 molar concentration and urea at .016 to .032, encouraged growth. Urea was an excellent nutrient, but sharply inhibited at stronger concentrations. Most amino acids as the sole source of nitrogen failed to support growth. With favorable nitrate, a number of amino acids were employed at pH 6.0. Arginine, probably because of its relation to urea, encouraged growth over a wide range. All others tested inhibited growth at about .001 *M*. While showing inhibition at this level, alanine, glutamic acid, and aspartic acid encouraged growth at about .064 *M*. The well-known facility of these three amino acids for transamination indicated that such reactions were changing the content of the medium from an unfavorable to a favorable balance at about .064 *M* concentration. In fact, transaminase was found in the tissue [Eberts, Burris, & Riker, (26)]. No doubt inhibition by various amino acids might be corrected by a balance with other amino acids, as has been shown with bacteria. With these three amino acids, the tissues may have supplied their own correction. The inhibition at about .001 *M* was not caused by interference with the utilization of nitrate. Ammonia was an intermediate, as shown with $N^{15}$-labeled nitrate. The ready use of urea might explain the presence of urease in various plant tissues where no urea was detected. The striking inhibition at about .001 *M* has indicated that rather small disturbances in the balances of different amino acids might be used to inhibit growth. The enormous numbers of possibilities with combinations of amino acids and related substances give hope for finding one that would inhibit tumor growths and even pathogens without being harmful to healthy tissue.

Further study of nitrogen metabolism [Eberts, Burris & Riker (26)] showed that both nitrate and amino acid were used when both were present. When sunflower tissue was incubated for short periods in a medium containing $N^{15}$-labeled DL-alanine or DL-glutamic acid, the distribution of the $N^{15}$ among the amino acids isolated from a tissue hydrolyzate differed somewhat with the amino acid and with the concentration at which it was supplied. In general, however, glutamic acid was the most active amino acid and alanine, aspartic acid, threonine, serine, methionine, and phenylalanine also were highly active.

Common sources of carbon were examined by Hildebrandt & Riker (35) with gall tissue cultures from marigold, Paris daisy, periwinkle, tobacco and sunflower. Great variations appeared between the kinds of tissue, between the sources of carbon, and their concentrations. For example, marigold tissue grew poorly at 1 per cent levulose and grew well at 4 per cent. The reverse was the case with periwinkle. Only periwinkle grew well on lactose or galactose. Since sugars might be fermented to produce alcohols or acids, some representatives were tried.

Various alcohols, with the same tissues, supported little, if any, growth [Hildebrandt & Riker (36)]. Moreover, with 2 per cent sucrose, ethanol inhibited marigold tissue at 0.25 per cent. Butanol inhibited all the tissues except periwinkle at 0.125 per cent. Propanol inhibited all the tissues at .06 per cent. Some of the higher alcohols were more selective.

Various organic acids at pH 6.0 with the same tissues supported little.

if any, growth as the sole source of carbon [Hildebrandt, Riker & Watertor (42)]. With 2 per cent sucrose, many of the acids were tolerated over a wide range of concentrations. Varying degrees of inhibition from partial to complete usually resulted as the concentrations of the acids were increased. Considerable specificity appeared as to acid, concentration, and species in relation to good, poor, or no growth. Acetic, formic, and propionic acids all either inhibited or stopped growth completely, even at 0.015 per cent. Some of the acids in the Kreb's cycle considerably improved growth when dilute. Doubtless certain acids were valuable through transamination for the development of amino acids. The role of organic acids in plant metabolism has been reviewed by Burris (19).

Various organic acids were produced by periwinkle, marigold, and sunflower tissue cultures in a medium with sucrose as the only added organic substance [Scott, Burris & Riker (85)]. *Alpha*-ketoglutamic acid appeared in unusually large amounts in periwinkle and sunflower tissues. Differences were observed in the quantities of other acids produced from species to species.

Such studies have provided a great deal of valuable background information for various basic studies. They have shown also the importance of using not only a range of concentrations, but also various species in studies of the effects of nutrients and metabolic substances. An approximation of the amounts of many simple amino and organic acids which encourage growth and, more important, those which inhibit growth has been indicated. Since certain metabolic substances have been used which were not in the common metabolic cycles, some alternate cycles might be indicated. The results have several other implications [Hildebrandt, Riker & Watertor (42)]: (*a*) The inhibition by a slight imbalance from a number of amino acids, alcohols, and organic acids has been apparent. The chances seem favorable that such metabolic materials with a suitable balance may be involved when plant tumor cells eventually are inhibited without injury to the healthy cells. (*b*) These inhibitions might have some association with differentiation and the development of maturity of tissues and the lack of further cell division. (*c*) The strong action of formic, acetic, propionic, butyric, and oxalic acids against plant tissues may deserve further consideration concerning the products of plant pathogens in relation to their pathogenicity. *Penicillium oxalicum,* Currie and Thom, destroyed corn with oxalic acid [Johann, Holbert & Dickson (45)]. (*d*) In the other direction, the presence of such acids in plant tissues may discourage various plant pathogens, e.g., protocatechuic acid in onions [Walker, Link & Angel (97)], and thus provide the host cells with resistance or even immunity.

Plant tissue cultures from insect-induced galls and from homologous healthy tissue appeared to offer opportunities for the study of interesting abnormal types of differentiation. Tissues from more than a dozen different types of insect galls were established *in vitro* by Hildebrandt, Riker & Klemmer (40). The requirements of gall and normal tissues were compared for coconut milk supplements and various combinations of growth substances

including kinetin and naphthaleneacetic acid. Suitable temperature and acidity have proved to be important for isolated insect gall and normal tissues from elm, grape, poplar, and willow [Pelet & Hildebrandt (78)].

The action of chloramphenicol, polymixin, and oxytetracycline on crown gall of marigold tissue was studied in culture. The tissue culture was inhibited with less polymixin than the crown gall bacteria, but withstood more oxytetracycline [Klemmer, Riker & Allen (50)]. The gall inhibition induced in whole plants by chlortetracycline, oxytetracycline, and chloramphenicol was caused largely by antibiotic activity against the crown gall tissue cells rather than against the causal bacteria.

### Single Cell Cultures

The long-recognized desire to grow higher plants from individual cells was fulfilled by Muir, Hildebrandt & Riker (66). Marigold and tobacco tumor tissues were grown in liquid shake cultures, where many individual cells occurred free in the medium. When the cultures were at the most vigorous stage of growth, a few drops of the medium containing strongly growing cells were spread aseptically over agar in a Petri dish. Under a dissecting microscope, individual cells were observed and lifted away with a microspatula. A cell was placed aseptically on a small (8 × 8 mm.) piece of rough-surfaced filter paper resting on a vigorously growing mass of tissue, which acted as a nurse or host. Growth of the isolated cells averaged about 8 per cent but sometimes the average rose to 50 per cent. They were observed as frequently as desired either with a magnifying glass or a dissecting microscope. Thus, the technique has been developed for establishing single-cell clones. Torrey (94) has made microscopic observations of single cells through a number of divisions.

The submerged cultivation of pole bean cells in an agitator provided cultures of single cells in suspension [Nickell (70)], permitting important variations in experimental procedures. This is not to be confused with single-cell cultures described above, which were the progeny of a mechanically picked and isolated single cell.

The most favorable growth for producing the isolations was found in the basic liquid sunflower or tobacco medium [Hildebrandt, Riker & Duggar (39)] at pH 6.0. After aeration on a fast shaker for about 3 weeks, the cells were in a vigorous condition [Muir, Hildebrandt & Riker (67)], suitable for isolation.

The possibility of cells growing through the filter paper was examined in various ways, always with negative results. Since the plant cells have no pseudopods or dendrites, the chances were quite remote. In some cases, marigold single cells grew on filter paper over sunflower tissue. The two were visually distinguishable. In other cases, the single cells grew on a corner of the filter paper well away from the tissue below. In no case were cells ever observed except at the location on the filter paper where they were placed.

Clones derived from single cells have shown considerable variations

in rate of growth, color, surface appearance, friability, and responses to media of different compositions [Muir, Hildebrandt & Riker (67)]. Some cells grew well only while nourished by living tissue; even a large mass of cells taken from them failed on the best agar cultures available. The demonstration that the various cells in the tumor mass are a heterogeneous mixture has answered an important question.

Certain of the clones with suitable balances of auxin and kinetin, respectively, produced roots or shoots. Morel (63) has worked with such shoots and has grown an entire normal plant. All the steps in the development of a whole plant from an isolated cell have been taken, although not yet in sequence.

The applications of the single-cell technique are numerous. For example, the different components in the crown gall mixture of cells can be analyzed. Cells from tissues with different degrees of differentiation can be studied. Among other workers, Gautheret (28) has described the variability of tissues in culture. With single-cell clones, one can determine whether those cells which are best adjusted to the medium used, outgrow the others. Furthermore, the variability of a particular clone can be determined. Tiny embryos heretofore impossible to culture can be tried and eventually, no doubt, a single fertilized ovum will be grown and manipulated. Embryo culture has been reviewed by Rappaport (79).

These investigations with both normal and tumor tissues have provided excellent backgrounds for various additional studies. Among these, mentioned by White in discussions, may be listed the following: (*a*) cytological work to find evidence of polyploidy, aneuploidy, mutations, and a cellular basis for anergie; (*b*) the initiation of different kinds of tissues; (*c*) morphogenesis; (*d*) population "genetics" of the tumor mass to determine what kinds of cells are present and how they develop; and (*e*) cell and tissue metabolism, including requirements, interactions, stimulation and inhibition.

The media have been sufficiently developed that some tissues grow quite rapidly, doubling their wet weight in several days. With further research, this rate of growth doubtless will be increased. Since these higher plant tissues now can be grown by means of the techniques used for microorganisms, possibly they can be used in the same manner as microorganisms for the production of special metabolic products. Even the production of fresh food in out-of-the-way places and from plants not ordinarily considered edible, becomes an interesting possibility.

## Use with Other Plant Diseases

The development of tissue culture technique has opened the way for a number of investigations in which the causal agents of plant disease may be involved. Some of these are concerned with the intimate relationships of the host and parasite at the cellular level. Others are concerned with the metabolism of the host in comparison with that of the pathogen. Such com-

parative studies give great promise of clarifying basic problems of pathogenicity and disease resistance.

With complete chemical and physical control over the metabolic activity of host tissue in culture, many new opportunities appear. This is especially noteworthy with respect to viruses, obligate fungi, and nematodes which do not increase on ordinary culture media.

*Virus diseases.*—A number of different viruses have been successfully cultured in isolated plant tissue growing *in vitro*. White (99) isolated and grew tomato roots infected with aucuba mosaic and tobacco mosaic virus (TMV). Other early studies by Augier de Montgremier, Limasset & Morel (1) described successful cultivation of virus in tobacco tissue cultures. Callus cultures established by Morel (61) from several species were infected, respectively, with TMV, potato virus X, and potato virus Y. TMV was also transmitted to established virus-free tissue cultures by mechanical rubbing. Culture growth and TMV concentrations in single cell clones of normal tobacco callus studied recently [Hildebrandt (33)] have shown large differences in the amount of TMV they carried.

The virus-infected tissue cultures provided interesting materials for the clarification of nutritional and other environmental requirements in host tissue growth and virus multiplication. Nickell (69) found that the virus tumor tissue from *Rumex* had a high optimum phosphate requirement and was stimulated by ribonucleic acid (RNA) hydrolyzate and inhibited by deoxyribonucleic acid (DNA) hydrolyzates. The tissue also released to the medium the enzyme $\alpha$-amylase [Brakke & Nickell (10)].

Tumor tissue of *Rumex acetosa* incited by and containing the wound tumor virus was first cultured by Black (7) in 1944. The virus was transmitted to *Rumex* by grafting slices of cultured *Rumex* tumor tissue. Recent grafts failed to demonstrate the presence of the virus in the cultured *Rumex* tumor originally isolated in 1944. A wound tumor virus antiserum [Black & Brakke (8)] also failed to show a virus antigen in extracts of the tissue cultures.

Tobacco mosaic virus multiplication has been influenced in tobacco tissue cultures by environmental conditions. Temperature and acidity were critical items in the amount of growth of Morel's TMV-infected tissues [Hildebrandt (33)]. The action of various nitrogen compounds on TMV multiplication in tobacco tissue cultures was examined by Segretain & Hirth (86). The virus concentration per unit wet weight of tissues increased with increasing levels of nitrate and potassium and decreased with increasing levels of phosphate in the culture medium [Hildebrandt (33)]. The virus also multiplied in cultures on media containing for nitrogen, respectively, sodium nitrite or ammonium chloride, sulfate, nitrate, succinate, or tartrate. With growth of the host tissue culture, the virus multiplied. With RNA as a nitrogen source, the growth of normal tobacco tissue in culture was encouraged or inhibited, respectively, at concentrations roughly corresponding to the amounts in thick dark green or in thin yellow portions of a tobacco

leaf showing pronounced mosaic [Hildebrandt, Riker & Muir (41)].

Many substances have been tested for antiviral activity with TMV-infected callus tissue by Kutsky (54), Kutsky & Rawlins (55), Kassanis (46), and by Hildebrandt (33). So far, 6-methylpurine has inhibited the virus more than the other chemicals tested and with little or no injury to the host tissue [Kurtzman *et al.* (53)].

Since the virus lags behind the tip of a rapidly-growing sprout, Morel & Martin (64) have made tissue cultures of such tips and grown whole plants. By this method they have freed potatoes, dahlias, and carnations from virus; a technique having important economic applications.

*Bacterial diseases.*—Certain nonpathogenic bacteria are able to live in association with tissues in culture without any obvious effects. Since many of them fail to grow on the synthetic media, it has been necessary to use a medium particularly rich in nutrients to detect their presence, as described by Robbins & Hervey (84). To be sure of the technique, such examination of the cultures has been found desirable each time the stock cultures are transferred or prepared for experimental use. This is in accord with occasional reports that bacteria may sometimes occur in apparently healthy plant tissues. However, if pathogenic soft rot bacteria are introduced into the cultures, Volcani, Riker & Hildebrandt (96) have shown that the tissues may disintegrate in a day or two.

*Fungus diseases.*—The growth of obligate parasites in tissue cultures has been a splendid step forward. Morel (60, 61) explored this valuable technique with *Plasmopara* on grape tissue. More recently (correspondence) he has cultivated the rust, *Puccinia,* on snapdragon.

The cultivation of rusts with tissue cultures was encouraged by Morel's success with the mildews. At his suggestion, studies were made with *Gymnosporangium* and other rust fungi. Early success was reported by Hotson & Cutter (43) and by Cutter (22). Since then Cutter (correspondence) has tried 41 rusts on 60 vascular plants which were systemically invaded. He isolated seven strains of *Gymnosporangium juniperi-virginianae* which eventually grew in the complete absence of the host tissue culture and infected their alternate rosaceous host. Five strains of *Uromyces caladii* were isolated from *Arisaema triphyllum,* of which three grew after a time in the absence of the host tissue culture and reinfected their host. Cutter isolated one strain of *Puccinia malvacearum* from *Althaea rosea* which also proved able to grow away from the host culture and to infect its host. He considered that the strains isolated were not typical of the general populations of these fungi, because of the rarity of successful isolations and idiosyncrasies following inoculations. These aberrant strains appeared at random. He thought that the successful isolation of only three species reflected the ease with which the infected host tissue could be established and maintained in tissue culture rather than of any inherent propensities on the part of the rust species.

The flax rust fungus, *Melampsora lini* was grown in culture on frag-

ments of surface sterilized flax leaves from the cotyledons [Ledingham & Turel (56)]. Beginning with Knop's basic medium, various supplements were added. Some compounds stimulated the callus and inhibited the rust, while others did the reverse. Uredospores and teliospores were observed frequently.

Tissue cultures from stem, leaf, and tuber of potato have been examined by Young *et al.* (106). Many cultures contained a mycorrhiza-like fungus comparable to that found in the plant. The fungus could not be isolated from the plant or cultured tissue, but tissue free of the mycorrhiza-like structure was secured by microdissection. The relationship of the mycorrhiza-like fungus to tuberization was interesting.

Three natural extracts obtained from two insect larvae and a Basidiomycete attacking oaks were employed on tissue cultures by Jacquiot (44). They induced responses like those from auxin.

*Nematode diseases.*—Important recent developments in nematology have, of necessity, been somewhat limited because of the inability to secure quantities of pathogenic nematodes free of saprophytic forms. With tissue cultures, a beginning has been made by Darling, Faulkner & Wallendal (25) to develop "pure" cultures of nematodes. Eggs have been secured aseptically from a single nematode and placed on potato, carrot, clover, and tobacco tissue growing in culture. The eggs have hatched promptly and the nematodes have increased to large populations in three to four months. This opens up a number of important techniques concerned both with the pathogenicity and physiology of these parasites and the resistance of the host plants.

## Applications

The gradual development of tissue culture technique has indicated procedures useful in almost every field of experimental biology, particularly in cytology, histology, cell physiology, cell pathology, susceptibility and resistance, and the study of tumors and viruses. Parker (77) prepared a long list of applications for experimental biology and medicine. The investigations discussed earlier make possible numerous applications in botanical fields, many of which contribute to fundamental biology. It is possible, for example, to study the nutrient requirements of cell types; the nature and various forms of energy-producing reactions and other biochemical changes occurring in the culture system; the physiological and functional characteristics of the various cell types which comprise the individual; the utilization of isotopically labelled substances incorporated in the medium; the mitochondria and other cytoplasmic inclusions; the effects of irradiation, temperature, and other physical agents on the cells; the specific action of known chemicals on different cell types and organ structures; the interactions of various metabolic substances which influence differentiation and organ formation; the properties of pure strains or clones derived from single-cell isolation; the similarities and differences between normal cells

and tumorous cells of similar origin; the transformation of normal to tumorous cells; the conditions necessary for the propagation of viruses; the usefulness of modified cell physiology in inhibiting viruses; the cultivation and study of obligate pathogens such as mildews, rusts, and nematodes; the reactions of host cells to pathogenic microorganisms and to their metabolic products; the pathogenic mechanisms of microorganisms; and the mechanism of disease susceptibility or resistance of host cells.

## LITERATURE CITED


1. Augier de Montgremier, H., Limasset, P., and Morel, G., *Compt. rend.*, **227**, 606–8 (1948)
2. Ball, E., *Growth*, **14**, 295–325 (1950)
3. Ball, E., *Growth*, **17**, 169–82 (1953a)
4. Ball, E., *Growth*, **17**, 215–28 (1953b)
5. Ball, E., *Année biol.*, **31**, 81–105 (1955)
6. Bitancourt, A. A., *Année biol.*, **30**, 361–69 (1954)
7. Black, L. M., *J. Natl. Cancer Inst.*, **19**, 663–85 (1957)
8. Black, L. M., Brakke, M. K., *Phytopathology*, **44**, 482 (1954)
9. Bove, J., and Morel, G., *Rev. gén. botan.*, **64**, 34–39 (1957)
10. Brakke, M. K., and Nickell, L. G., *Arch. Biochem. Biophys.*, **32**, 28–41 (1951)
11. Braun, A. C., *Am. J. Botany*, **30**, 674–77 (1943)
12. Braun, A. C., *Am. J. Botany*, **34**, 234–40 (1947)
13. Braun, A. C., *Phytopathology*, **41**, 963–66 (1951)
14. Braun, A. C., *Cancer Research*, **16**, 53–56 (1956)
15. Braun, A. C., *J. Natl. Cancer Inst.*, **19**, 753–69 (1957)
16. Braun, A. C., *Proc. Natl. Acad. Sci. U. S.*, **44**, 344–49 (1958)
17. Braun, A. C., and Stonier, T., *Protoplasmatologia* (In press, 1958)
18. Braun, A. C., and White, P. R., *Phytopathology*, **33**, 85–100 (1943)
19. Burris, R. H., *Ann. Rev. Plant Physiol.*, **4**, 91–114 (1953)
20. Caplin, S. M., *Botan. Gaz.*, **108**, 379–93 (1947)
21. Caplin, S. M., and Steward, F. C., *Nature*, **163**, 920–25 (1949)
22. Cutter, V. M., *Trans. N. Y. Acad. Sci.*, **14**, 103–8 (1951)
23. Czosnowski, J., *Poznan Soc. Friends Sci., Dept. Math. Natl. Sci., Biol. Sec.*, **13**, 31–38 (1952a)
24. Czosnowski, J., *Poznan Soc. Friends Sci., Dept. Med., Publ. Sect. Exptl. Med.*, **13**, 206–8 (1952b)
25. Darling, H. M., Faulkner, L. R., and Wallendal, P., *Phytopathology*, **47**, 7 (1957)
26. Eberts, F. S., Burris, R. H., and Riker, A. J., *Plant Physiol.*, **29**, 1–10 (1954)
27. Gautheret, R. J., Ed., *Année biol.*, **30**, 262–460 (1954); **31**, 1–241 (1955)
28. Gautheret, R. J., *Année biol.*, **31**, 145–71 (1955a)
29. Gautheret, R. J., *Ann. Rev. Plant Physiol.*, **6**, 433–84 (1955b)
30. Gautheret, R. J., *J. Natl. Cancer Inst.*, **19**, 555–73 (1957)
31. Henderson, J. H. M., and Bonner, J., *Am. J. Botany*, **39**, 444–51 (1952)
32. Henderson, J. H. M., Durrell, M. E., and Bonner, J., *Am. J. Botany*, **39**, 467–73 (1952)
33. Hildebrandt, A. C., *Proc. Natl. Acad. Sci. U. S.*, **44**, 354–63 (1958)
34. Hildebrandt, A. C., and Riker, A. J., *Am. J. Botany*, **34**, 421–27 (1947)
35. Hildebrandt, A. C., and Riker, A. J., *Am. J. Botany*, **40**, 66–76 (1953)

36. Hildebrandt, A. C., and Riker, A. J., *Cancer Research*, **15**, 517–22 (1955)
37. Hildebrandt, A. C., Riker, A. J., Duggar, B. M., *Am. J. Botany*, **32**, 357–61 (1945)
38. Hildebrandt, A. C., Riker, A. J., and Duggar, B. M., *Am. J. Botany*, **33**, 591–97 (1946a)
39. Hildebrandt, A. C., Riker, A. J., and Duggar, B. M., *Cancer Research*, **6**, 368–77 (1946b)
40. Hildebrandt, A. C., Riker, A. J., and Klemmer, H. W., *Plant Physiol.*, **31**, Suppl., 38 (1956)
41. Hildebrandt, A. C., Riker, A. J., and Muir, E., *Plant Physiol.*, **32**, 231–36 (1957)
42. Hildebrandt, A. C., Riker, A. J., and Watertor, J., *Phytopathology*, **44**, 422–28 (1954)
43. Hotson, H. H., and Cutter, V. M., *Proc. Natl. Acad. Sci. U. S.*, **37**, 400–3 (1951)
44. Jacquiot, C., *Année biol.*, **30**, 457–59 (1954)
45. Johann, H., Holbert, J. R., and Dickson, J. G., *J. Agr. Research*, **43**, 757–90 (1931)
46. Kassanis, B., *Virology*, **4**, 5–13 (1957)
47. Klein, R. M., Rudnick, D., Ed., *Rhythmic and Synthetic Processes in Growth*, 31–58 (Princeton Univ. Press, Princeton, N. J., 1957a)
48. Klein, R. M., *Proc. Natl. Acad. Sci. U. S.*, **43**, 956–60 (1957b)
49. Klein, R. M., and Vogel, H. H., *Plant Physiol.*, **31**, 17–22 (1956)
50. Klemmer, H. W., Riker, A. J., and Allen, O. N., *Phytopathology*, **45**, 618–25 (1955)
51. Kulescha, Z., *Année biol.*, **30**, 319–27 (1954)
52. Kulescha, Z., and Gautheret, R. J., *Compt. rend.*, **227**, 292–94 (1948)
53. Kurtzman, R., Hildebrandt, A. C., Riker, A. J., and Burris, R. H., *Phytopathology*, **47**, 527 (1957)
54. Kutsky, R., *Science*, **115**, 19–20 (1952)
55. Kutsky, R., and Rawlins, T. E., *J. Bacteriol.*, **60**, 763–66 (1950)
56. Ledingham, G. A., and Turel, F., *Intern. Wheat Rust Conf., 3rd Conf.*, 133 (Mexico, D. F., Mexico, 1956)
57. Locke, S. B., Riker, A. J., and Duggar, B. M., *J. Agr. Research*, **57**, 21–39 (1938)
58. Miller, C. O., and Skoog, F., *Am. J. Botany*, **40**, 768–73 (1953)
59. Miller, C. O., Skoog, F., Okumura, F. S., Von Saltza, M. H., and Strong, F. M., *J. Am. Chem. Soc.*, **78**, 1375–80 (1956)
60. Morel, G., *Compt. rend.*, **218**, 50–52 (1944)
61. Morel, G., *Ann. épiphyt.*, **14**, 123–234 (1948)
62. Morel, G., *Compt. rend.*, **230**, 1099–1101 (1950)
63. Morel, G., *Rev. gén. botan.*, **63**, 314–24 (1956)
64. Morel, G., and Martin, C., *Intern. Hort. Congr., 14th Congr.*, **1**, 303–10 (Hague-Scheveningen, The Netherlands, 1955)
65. Morel, G., and Wetmore, R. H., *Am. J. Botany*, **38**, 141–43 (1951)
66. Muir, W. H., Hildebrandt, A. C., and Riker, A. J., *Science*, **119**, 877–78 (1954)
67. Muir, W. H., Hildebrandt, A. C., and Riker, A. J. (In press, 1958)
68. Murray, M. R., and Kopech, G., *A Bibliography of the Research in Tissue Culture, 1884–1950* (Academic Press, Inc., New York, N.Y. 2 Vol., 1953)
69. Nickell, L. G., *Année biol.*, **31**, 107–21 (1955)

70. Nickell, L. G., *Proc. Natl. Acad. Sci. U. S.*, **42**, 848–50 (1956)
71. Niedergang-Kamien, E., and Skoog, F., *Physiol. Plantarum*, **9**, 60–73 (1956)
72. Nitsch, J. P., and Nitsch, C., *Am. J. Botany*, **43**, 839–51 (1956)
73. Nitsch, J. P., and Nitsch, C., *Am. J. Botany*, **44**, 555–64 (1957)
74. Nobecourt, P., *Ber. schweiz. botan. Ges.*, **65**, 475–80 (1955)
75. Okumura, F. S., Von Saltza, M. H., Strong, F. M., Miller, C. O., and Skoog, F., *Am. Chem. Soc., 28th Meeting* (Minneapolis, Minn., 1955)
76. Parker, R. C., *Methods of tissue culture*, 2nd ed. (Paul B. Hoeber, Inc., New York, N.Y., 294 pp., 1950)
77. Parker, R. C., *Encyclopedia Britannica*, **1**, 964d-964Bd (1958)
78. Pelet, F., and Hildebrandt, A. C., *Phytopathology*, **47**, 531 (1957)
79. Rappaport, J., *Botan. Rev.*, **20**, 201–25 (1954)
80. Reinert, J., and White, P. R., *Physiol. Plantarum*, **9**, 177–89 (1956)
81. Rier, J. P., and Henderson, J. H. M., *Plant Physiol.*, **32**, 494–97 (1957)
82. Riker, A. J., *Growth Symposium, 4th Symp.*, **6**, 105–17 (1942)
83. Riker, A. J., and Gutsche, A., *Am. J. Botany*, **35**, 227–38 (1948)
84. Robbins, W. J., and Hervey, A., *Science*, **114**, 322–23 (1951)
85. Scott, R. W., Burris, R. H., and Riker, A. J., *Plant Physiol.*, **30**, 355–60 (1955)
86. Segretain, G., and Hirth, L., *Compt. rend.*, **147**, 1042–43 (1953)
87. Skoog, F., *J. Natl. Cancer Inst.*, **19**, 493–94 (1957)
88. Skoog, F., and Miller, C. O., *Symposia Soc. Exptl. Biol. No. 11*, 118–31 (1957)
88a. Skoog, F., and Tsui, T., *Am. J. Botany*, **35**, 782–87 (1948)
88b. Skoog, F., and Tsui, T., in Skoog, F., Ed., *Plant Growth Symposium*, 263–85 (Univ. Wisconsin Press, Madison, Wis., 1951)
89. Steward, F. C., and Caplin, S. M., *Année biol.*, **30**, 395–98 (1954)
90. Steward, F. C., and Pollard, J. K., *Ann. Rev. Plant Physiol.*, **8**, 65–114 (1957)
91. Steward, F. C., and Shantz, E. M., *Année biol.*, **30**, 399–410 (1954)
92. Steward, F. C., and Shantz, E. M., *Plant Physiol.*, **34** (In press, 1959)
93. Struckemeyer, B. E., Hildebrandt, A. C., and Riker, A. J., *Am. J. Botany*, **36**, 491–95 (1949)
94. Torrey, J. G., *Proc. Natl. Acad. Sci. U. S.*, **43**, 887–91 (1957)
95. Van Lanen, J. M., Baldwin, I. L., and Riker, A. J., *J. Bacteriol.*, **63**, 723–34 (1952)
96. Volcani, Z., Riker, A. J., and Hildebrandt, A. C., *Phytopathology*, **43**, 92–94 (1953)
97. Walker, J. C., Link, K. P., and Angel, H. R., *Proc. Natl. Acad. Sci. U. S.*, **15**, 845–50 (1929)
98. Wetmore, R. H., in Rudnick, D., Ed., *Cellular Mechanisms in Differentiation and Growth*, 173–90 (Princeton Univ. Press, Princeton, N.J., 1956)
99. White, P. R., *Phytopathology*, **24**, 1003–11 (1934)
100. White, P. R., *Bull. Torrey Botan. Club*, **66**, 507–13 (1939)
101. White, P. R., *A Handbook of Plant Tissue Culture*, (Jacques Cattell Press, Lancaster, Pa., 227 pp., 1943)
102. White, P. R., *Ann. Rev. Plant Physiol.*, **2**, 231–44 (1951)
103. White, P. R., *The Cultivation of Animal and Plant Cells*, (The Ronald Press Co., New York, N.Y., 239 pp., 1954)
104. White, P. R., Ed., *J. Natl. Cancer Inst.*, **19**, 467–843 (1957)
105. White, P. R., and Braun, A. C., *Cancer Research*, **2**, 597–617 (1942)
106. Young, D. A., Hildebrandt, A. C., Rieman, G. H., and Cooper, D. C., *Phytopathology*, **43**, 490 (1953)

# FACTORS DETERMINING THE PATHOGENICITY OF STAPHYLOCOCCI[1]

By John E. Blair
*Laboratory Division, Hospital for Joint Diseases, New York, New York*

During the past few years infections due to the staphylococci have attained the unenviable distinction of being among the most prominent of bacterial diseases. The problem of staphylococcal infection is particularly serious in many hospitals, and is complicated by the fact that a large proportion of "hospital" strains are antibiotic-resistant. There is growing evidence that staphylococcal infections among the general population sometimes can arise from organisms transmitted by individuals who have acquired them while patients in the hospital.

The development of new, precise knowledge concerning the mechanisms of pathogenicity of the staphylococci and host-parasite relationships has lagged behind the rapid rise of the staphylococci to their present position of importance. Past investigations have contributed much valuable information toward a partial solution of some of these problems, but the bacteriologist, epidemiologist, and clinician now are faced with a multitude of questions that demand to be answered.

It can readily be demonstrated in the laboratory that pathogenic staphylococci elaborate a variety of toxins and enzymes, and these substances can be shown experimentally to produce certain definite effects in suitable living or *in vitro systems*. Several interesting and suggestive hypotheses have been formulated concerning the possible roles played by cellular constituents or products of staphylococci in the initiation of infection and the production of disease. The characterization of staphylococcal toxins and enzymes and their identification with a role in pathogenesis await their isolation in a highly purified state. This also is a prerequisite for a more precise study of the nature of immunity to staphylococcal infection. Fortunately, some progress is being made in this direction.

Recent experimental observations, while far from providing definitive answers to some of the problems, at least can serve as useful guides or points of departure for future investigations. It is the purpose of this article briefly to summarize some of the concepts that have been advanced concerning the pathogenesis of staphylococcal infection.

## Toxins

*Exotoxin.*—A potent toxic staphylococcal culture filtrate produces rapid death of rabbits and mice when injected intravenously, is highly necrotic

[1] The survey of the literature pertaining to this review was concluded in November, 1957.

when injected intradermally in rabbits, and hemolyzes rabbit and sheep erythrocytes. The culture filtrates of most toxigenic strains of *Staphylococcus aureus* exhibit these properties in approximately quantitative relationships, and they are neutralized quantitatively by antitoxin. Although opinions differ, it appears to be held by many investigators that the lethal, necrotic and hemolytic reactions are manifestations of the activity of a single toxic substance. While the possibility exists that a toxic filtrate may contain more than one factor responsible for these effects, it would seem to be significant that Pillemer & Robbins (1) found that all three properties were associated with a purified toxin which contained over 2000 Lf per mg. N.

The rapid death which follows the intravenous injection of potent toxin in mice or rabbits suggests a direct primary effect. When death occurs a few hours after administration of the toxin, it is conceivable either that time is required for the toxin to accumulate in susceptible tissues, or that secondary toxic substances are being formed in tissues that were damaged by the toxin. Rabbits that are injected intravenously with washed toxigenic staphylococci appear clinically normal for about 24 hr., but die with all the characteristic symptoms that are observed after the intravenous injection of a potent toxic filtrate (2). During the first few hours after injection the temperature is elevated, and at about 18 to 24 hr. electrocardiographic changes appear that are indicative of myocardial damage; the agonal period lasts only about 20 to 30 min. It would appear that, as the toxin is elaborated *in vivo*, its lethal effect is delayed until a critical threshold dose has accumulated.

Care must be used in applying the experimental observations to an interpretation of the role of toxin in the pathogenesis of infections in man. Relatively large doses of toxin (usually from 0.1 to 0.5 ml. per kilo of body weight) are required to produce the rapid lethal effect in mice and rabbits; this somewhat artificial situation may have little resemblance to the slow formation of toxin by staphylococci in the body. However, it seems probable that the lethal component of toxin may contribute to the rapidly fatal termination of some acute staphylococcal infections, especially in children and young adults. This would seem to have been true of the deaths in the Bundaberg series, in which 12 of 21 children died of acute toxemia within 34 hr. after they had received a prophylactic injection of diphtheria toxin-antitoxin contaminated by *S. aureus* (3). Kleiger & Blair have pointed out the close similarity between the manifestations of toxicity noted in experimental animals after the intravenous administration of toxin and the clinical symptoms of acute staphylococcal toxemia in children from whom highly toxigenic staphylococci were isolated (4). The fact that acute toxemia in the human subject can be controlled by adequate therapeutic doses of antitoxin, often with dramatic amelioration of the clinical symptoms, strongly suggests that the toxin may play a not inconsiderable role in the pathogenesis of this form of staphylococcal disease (5).

The exact contribution of toxin to the establishment of a localized in-

fection is less clear. The intense necrosis that follows the intradermal injection of potent toxin in rabbits and the common observation that many, if not all, strains of staphylococci from cutaneous lesions are toxigenic (6) have led to the assumption that toxin may be important to the initiation of infection in the intact skin. Elek (7) produced purulent lesions in the skin of human volunteers with washed toxigenic staphylococci and pointed out that multiplication of the cocci is necessary to the establishment of the lesion.

*Hemolysins.*—At least three antigenically distinct staphylococcal hemolysins have been identified, and designated as α-, β- and δ-hemolysins, respectively (8, 9, 10). The α-hemolysin, which lyses rabbit, but not human or sheep erythrocytes, has been so frequently associated with the lethal and necrotizing factors in toxic culture filtrates that the terms α-lysin and α-toxin are often used interchangeably. While complete correlation of the production of α-hemolysin with pathogenicity of staphylococci is claimed by some investigators, others have reported that a varying proportion of strains from human lesions produce no α-lysin. The occurrence of intravascular hemolysis, serosanguinous exudates in body cavities, or hemorrhages in the organs or serous linings of cavities following the injection of toxic culture filtrates into rabbits has been described by many investigators [cited by Blair (11)]. However, the proved existence of some pathogenic strains that do not produce α-hemolysin, and the fact that the effect of α-hemolysin on human erythrocytes is negligible, would seem to indicate that this hemolysin per se, is of little importance in the pathogenesis of staphylococcal infection in man.

No pathogenic role has been demonstrated for δ-hemolysin, which lyses the red blood cells of rabbits, sheep, and man. It is produced by over 90 per cent of human pathogens, usually in association with α-lysin, and it occurs as the only hemolysin in about four per cent. Zones of lysis on blood agar plates that actually were produced by δ-hemolysin probably have sometimes been attributed to α-hemolysin, and form the basis for claims that all pathogenic staphylococci are α-hemolytic. The characteristics and relationships of α-, β-, and δ-hemolysins have been described by Elek & Levy (12).

The β-hemolysin, which lyses sheep erythrocytes but not those of rabbits or man, is produced chiefly by strains of animal origin. Only a small proportion of human pathogens produce the lysin, which is always associated in these strains with α-lysin or δ-lysin, or both. A hemolytic factor, produced by coagulase-negative strains of staphylococci and designated as ε-hemolysin, has been described by Elek & Levy (12). Its existence has been questioned by some investigators. The term, "hemolytic staphylococcus," is unacceptable as the designation of a pathogenic staphylococcus, and its use should be avoided.

Various suggestions have been made concerning the mechanisms of action of bacterial toxins (13). Some manifestations of toxicity, whether on tissue cells or blood cells, may be referable to an enzyme or enzyme-

inhibiting component of the toxin; it may act directly on other enzymes or on enzyme substrates, may compete with a vital enzyme or other metabolic factor, or may convert a normal substrate into a toxic substance. As is the case with many other bacterial toxins, the mechanisms of action of staphylococcal toxin are unknown; nor is it known whether toxin or a component plays a role in the normal respiration or metabolism of the cell.

*Enterotoxin.*—Staphylococcal enterotoxin is an entity distinct from other toxic substances produced by these microorganisms. Studies on enterotoxin have been made difficult by the lack of a suitable method of assay. While far from satisfactory, it is believed by Dack and his associates that monkey-feeding tests are the most reliable available (14). Studies on the chemistry of the toxin have yielded information concerning its properties but are not themselves useful for assay. Highly purified enterotoxin, prepared on a pilot-plant scale and subjected to modern methods of protein fractionation, has been reported by Bergdoll (15) to have the following properties: a water-soluble protein with a molecular weight of 15 to 25,000; iso-electric point near pH 8.5; a high percentage of lysine; resistance to trypsin; antigenicity. Attempts at further purification by electrophoresis on paper (15, 16) or starch (15), and chromatography with a cation-exchange resin have not been entirely satisfactory because of loss of activity (15). The gel-diffusion technique of Oudin has revealed several bands of precipitate, one of which was tentatively identified as that of enterotoxin-antienterotoxin and was used to follow the several steps of purification (17). By infrared spectrophotometry it was reported that the intensity of absorption in the region of 1100 to 1000 $cm.^{-1}$ was greater with an enterotoxic than with a nontoxic preparation (18).

Conclusive evidence of the mode of action of enterotoxin has not yet been produced (19). It has been suggested that the site of emetic action may be localized in the peripheral sensory structures of the viscera or in the forebrain. Increased tonus of isolated segments of rabbit intestine has been produced by enterotoxic preparations but may be caused by impurities in the preparations. No specific action has been demonstrated for several enzymatic systems; although apyrase is concentrated by the methods used to purify enterotoxin, it does not appear to be related to the toxin.

*Leucocidin.*—Leucocidin is produced in varying amounts by the majority of pathogenic staphylococci. Although it would appear to be formed independently of the lethal and necrotizing factors, both leucocidin and the "toxin" often are present in the same culture filtrates and both are capable of destroying leucocytes. The determination of leucocidic activity by the Neisser-Wechsberg method, which is based upon the capacity of living rabbit leucocytes to reduce methylene blue, led to the assumption that leucocidin and the toxic factors are identical. However, a distinction can be made by the examination of stained microscopic preparations of mixtures of culture filtrate and human blood; toxin has no action on human leucocytes, and any destructive action on the white cells may reasonably be

attributed to the action of leucocidin (20). Very recently it has been suggested that leucocidin may be identical with δ-hemolysin which, while apparently not possessing lethal properties, does nevertheless cause disruption of human leucocytes (21).

Definitive information concerning the exact role in leucocidin in the pathogenesis of staphylococcal infection is scarce. Leucocidin has not been prepared in a highly purified state, and studies of the activity of purified toxin have been confined to its α-hemolytic, necrotizing, and lethal properties. In studies on the phagocytosis of pathogenic staphylococci, the destruction of the polymorphonuclear cells after they have ingested the cocci has often been described, from which observation it has been concluded that the effect was the result of the action of leucocidin. However, this inference has rarely been supported by the demonstration that a specific staphylococcal factor was responsible.

While coagulase-positive staphylococci may be ingested by human leucocytes and can survive within the leucocytes for many hours, a steady fall is noted in the total leucocytic count, which becomes quite rapid after a few hours (22). It was suggested that leucocidin may affect those metabolic activities of the leucocytes that are responsible for the destruction and digestion of the cocci. Some support of this view was found in the observation that a few nonpathogenic staphylococci may withstand destruction for a short period if the leucocytes are killed immediately after the cocci have been ingested. The intraleucocytic survival of staphylococci was suggested earlier by Lyons (23), who attributed it to the possession of a capsule by young cocci. Upon ingestion the capsule was lost by both pathogenic and nonpathogenic cocci, but was reformed by the pathogens, which then brought about the destruction of the leucocytes, presumably through the action of leucocidin. Rogers & Tompsett (22) were unable to demonstate encapsulation of the cocci, even in the early growth phase studied by Lyons, and found that staphylococci survived in human leucocytes regardless of the age of the culture.

## Enzymes

*Coagulase.*—The almost complete correlation of the ability of staphylococci to clot blood plasma with their capacity to produce disease, and the corresponding absence of this property in nonpathogenic strains, has led to the assumption that it is closely related to the establishment of infection. It has been firmly established that the clotting of plasma by staphylococci involves the interaction of two factors: an enzyme, coagulase, which is elaborated by the cocci, and a component of plasma known as the coagulase-reacting factor, or CRF (24). A number of observations have been cited in explanation of the mode of action of coagulase and of the plasma factor.

It was shown by Smith & Hale (25) that the infectivity of staphylococci for various animal species is related to the susceptibility of the plasma of the species to clotting by the cocci. Guinea pigs, for example, are relatively

refractory to infection and their plasma is generally not coagulable by staphylococci. However, infection could be produced experimently in occasional animals whose plasma was clotted by certain strains of staphylococci (26). It was also possible to produce abscesses in an insusceptible animal by the subcutaneous injection of staphylococci at a site that had been prepared by the injection of coagulable plasma from another species. It was suggested that by laying down a fibrin barrier, coagulase plays a major role in the initiation and development of the staphylococcal lesion, after which other factors such as toxins come into action. It was also suggested by Hale & Smith (27) that a possible role of coagulase is to inhibit phagocytosis by the deposition of a fibrin coat around the cocci. In an *in vitro* system the number of coagulase-positive staphylococci ingested by rabbit or human polymophonuclear leucocytes was reported to be significantly lower than in a comparable system using coagulase-negative cocci. The reaction was specific, for in mixtures of leucocytes, coagulase-positive staphylococci and bacteria of some coagulase-negative species only the staphylococci were ingested.

One may question whether coagulase acts in the body in the same manner that it does *in vitro*. The rapid *in vivo* phagocytosis of coagulase-positive staphylococci in rabbits has been described by Rogers (28). Menkin & Walston (29) found no evidence to indicate that bacteria-free coagulase caused blockage of the lymphatics when injected intradermally in rabbits, and they concluded that the localization of staphylococcal infection was independent of the plasma-clotting factor. Fisher (30) did not find excessive intravascular clotting or thrombosis during the postmortem examination of 27 cases of staphylococcal septicemia; he also was unable to demonstrate intravascular clotting, either grossly, microscopically, or clinically following the intravenous injection into rabbits of large doses of staphylococcal cultures. No clots resulted from the intracutaneous, subcutaneous, or intrascrotal injections in rabbits of mixtures of staphylococci and plasma which clotted *in vitro* within 30 min. No clots were found after the intrapleural injection of similar mixtures. It is of interest that after no longer than 1.5 to 3 hr. in the pleural cavity, the mixture did not clot afterward in the test tube and no fibrinogen could be demonstrated; clotting did occur when the mixture was injected into the pleural cavity of a dead rabbit or if the animal died within 15 min. after injection. Lack & Wailling have found no evidence for *in vivo* clotting by coagulase (31).

It has been demonstrated that normal human serum has little bactericidal activity against coagulase-positive staphylococci (32, 33). It was postulated by Ekstedt (33) that the serum-resistant strains were aided in their growth by the protective action of some metabolic product which was not formed by serum-susceptible strains. Various substances formed by staphylococci, such as polysaccharides and α- and β-toxins, were found to have no protective effect, and it was concluded that the protective role was played by coagulase. It was assumed that coagulase acted upon a nonspecific antibacterial

factor in the serum to neutralize its inhibitory action. Crude protein fractions prepared from culture supernatants retained their coagulase activity and permitted the growth of coagulase-positive staphylococci when added to normal serum. A quantitative relationship existed between coagulase and its inhibitory action on serum, and a certain level of coagulase production appeared to be necessary to permit growth of staphylococci in normal serum. The growth of "weakly" coagulase-positive strains was inhibited by serum, as was that of coagulase-negative strains; however, less coagulase was required to permit the growth of weakly coagulase-positive strains, when added to normal serum, than was needed for coagulase-negative strains. The antibacterial factor of the serum appeared to exert its effect directly upon the cocci; the enzymatic nature of the action is suggested by the fact that calcium ions were necessary for the inhibitory effect. Further investigations are indicated in which highly purified coagulase is used, and the possible effects of anticoagulase or other inhibitors are taken into consideration.

From the suggestions contained in the work by Smith & Hale, and from their own impression that staphylococcal infections in infants assume a more diffuse form and not the characteristic localized abscesses that are seen in adults, Rammelkamp & Lebovitz (34) felt that possibly some difference in the ability of the individual to lay down a fibrin barrier might be correlated with age. To explain this difference they studied the content of the coagulase-reacting factors in individuals of various age groups. It was found that about 93 per cent of normal adult sera contained at least 1600 units of CRF; in children, 78 per cent of the sera contained 1600 units or more; and in infants only small amounts of CRF were present, with about half showing less than 400 units. It was found that the CRF increased with age, and that in adults it remained relatively constant over a considerable period of time. The high content of CRF in adult sera would appear to correlate well with the characteristic tendency of staphylococcal infections to localize in adults. It would be of interest to know whether the CRF varies in different clinical forms of staphylococcal infection in infants and young children. The CRF was found to be depressed in the early stages of such diseases as primary atypical pneumonia, infectious hepatitis, and leptospirosis, but rose again to the usual adult concentration during the later stages. It would be interesting to know whether secondary staphylococcal infections are particularly common in these diseases.

Tager & Lodge (35) and Duthie & Lorenz (36) have shown that the CRF content of plasma is higher than that of serum. This does not invalidate the CRF values reported for serum by Rammelkamp and Lebovitz; it suggests, however, that comparable studies on the plasma of individuals of various ages and disease conditions would be informative.

The existence of several antigenically distinct coagulases is indicated by the reports of Rammelkamp and his associates (37) and of Duthie (38). The relationship between the coagulases described by Rammelkamp and by

Duthie is not known. It would appear that in studies on the mechanism of action of coagulase or CRF a type of coagulase should be employed for which few human sera contain a specific inhibitor; a Type III coagulase was used by Rammelkamp in the studies cited above.

Progress toward an understanding of the mode of action of coagulase, and of the reacting factor, has been aided by methods of purification which permit their more exact characterization. However, because the mechanisms of physiologic clotting have not themselves been fully established, it should be borne in mind that observations bearing on the mechanisms of coagulase and CRF and their relation to the known factors of physiologic clotting, must now be only suggestive.

A 300-fold purification of coagulase has been effected by Tager (39) by precipitation with acetate buffer at pH 3.8 to 4, purification by several cycles of treatment with ethanol in the cold at controlled pH, and separation of impurities with low concentrations of ammonium sulfate. The purified product was not dialyzable through Visking membranes. Its nitrogen content was 14 to 16 per cent, and it was inactivated rapidly by crystalline trypsin and chymotrypsin, as well as by plasmin. It was heat labile, in contrast to the relative thermostability of crude coagulase. Chromatography of hydrolyzed coagulase indicated the presence of aspartic acid, glutamic acid, serine, glycine, threonine or lysine, arginine, hydroxyproline or tyrosine, histadine, and tryptophan or valine. The purified product was not completely homogeneous on electrophoresis or ultracentrifugation. Purified coagulase was highly active biologically; the clotting time was inversely proportional to the concentration of coagulase, some preparations reacting fully as rapidly as suitable preparations of thrombin-fibrinogen.

The maximum recovery of CRF was obtained in association with globulin fractions obtained by dilution and acid precipitation at pH 5.3 according to the Mellanby technique, or by precipitation with ammonium sulfate at concentrations between 33 and 50 per cent saturation (24). By controlled ethanol precipitation by the method of Cohn, the CRF activity was found to reside in globulin fractions III-1, III-2, and IV-1. Albumin (Fraction V) contained no CRF, nor did Fraction I (fibrinogen) after it was further purified (39).

Prothrombin and CRF exhibit a number of properties in common which suggest that they may be identical or at least closely related. Among the common properties are: similar inactivation by heat, similar absorption, parallel consumption during physiologic clotting, disappearance in rabbits treated with dicumarol or phenylindanedione and equal restoration by the anticoagulant effect of vitamin $K_1$, and acceleration of activity by crystalline trypsin (39). On the other hand, there are certain distinct differences between CRF and prothrombin: physiologic clotting, but not clotting by coagulase, is inhibited by heparin and by citrate or oxalate ions; the two factors are differently distributed in the various plasma fractions of Cohn; the plasma of certain animal species is notably deficient in CRF; and clot-

ting by coagulase is unaffected by the action of soybean trypsin inhibitor, to which the process of physiologic clotting is sensitive.

A further difference was demonstrated by Tager & Hales (40) who showed that CRF and prothrombin can be separated by the filtration of human plasma through Seitz filter pads. After five successive filtrations, the filtrates contain 50 per cent of CRF activity but essentially no prothrombin activity. Highly purified CRF was prepared from such filtrates by adsorption on a column of hyfloamphogel, washing with phosphate buffer at pH 8, elution with phosphate buffer at pH 8.6, and precipitation in the cold with ammonium sulfate in concentrations of 33 and 45 to 50 per cent saturation (39).

Upon ultracentrifugation, purified human prothrombin showed much more rapid sedimentation than purified CRF; paper electrophoresis indicated that CRF activity corresponded to localization between the beta and gamma globulins, while that of prothrombin was concentrated in the $alpha_2$ and $alpha_1$ globulin zones (39). Tager has concluded that the CRF activity of plasma is represented by two factors; one of these, associated with large molecules, is presently indistinguishable from prothrombin and the other, associated with small molecules, shows no prothrombin activity but presumably is derived from prothrombin (39).

The exact mechanism of coagulase activity is undetermined. No evidence of proteolysis has been found (39). The suggestion that coagulase may be related to an arginase (41) would seem to be refuted by Sherry, who did not find evidence that coagulase could split synthetic arginine or lysine esters [cited by Tager (39)]. No correspondence of properties has been found between CRF and suspensions of human platelets or various accelerators, nor has confirmation been obtained of the claim that CRF is identical with antihemophilic globulin (39). It is of interest that the electron microscopic examination of fibrin produced by the action of purified coagulase, CRF, and human fibrinogen showed some fibers having the same range of periodicity as fibrinogen and some fibers with coarser striations (39).

*Fibrinolysin.*—The ability of staphylococci to dissolve fibrin clot is restricted essentially to coagulase-positive strains of human origin. With only a few exceptions (31), staphylococci which produce β-hemolysin, and are chiefly of animal origin, are nonfibrinolytic. Not all coagulase-positive strains are fibrinolytic; in a small series, approximately 81 per cent were found by Fisher (42) to dissolve human plasma or fibrinogen clots, while among over 400 cultures Lack & Wailling (31) reported that about 70 per cent were fibrinolytic. Lack (43) has suggested that the active principle is not a true fibrinolysin, but, rather, a staphylokinase which activates plasminogen. The lysis of fibrin clot would not appear to be due to a protease (31). The active principle is present in precipitates obtained by treating culture filtrates with alcohol (42); no attempts appear to have been made to effect a high degree of purification.

Little information is available concerning a possible role of fibrinolysin in the pathogenesis of staphylococcal infection. It has been suggested that the potential danger of a staphylococcus is greater if it is capable of dissolving the fibrin barrier and so spreading the infection (42); it has also been suggested that small emboli may be liberated by fibrinolysin from an infected thrombus, to lodge elsewhere in the body and establish metastases (42, 44).

*Hyaluronidase.*—In common with several other Gram-positive bacteria, the majority of pathogenic staphylococci elaborate an enzyme, hyaluronidase, which breaks down hyaluronic acid, a mucopolysaccharide that is a constituent of the intracellular ground substance of many body tissues. Hyaluronidase has been identified with the "spreading factor" described by Duran-Reynals.

As is the case in infections due to other hyaluronidase-producing bacteria, the role played by this enzyme in the pathogenesis of staphylococcal infection is a matter of conjecture. It might reasonably be supposed that it serves to enhance the invasion of tissues and dispersion of the cocci or diffusion of their toxic products. During the initiation of a naturally acquired infection, the number of staphylococci that originally come in contact with the host probably is not great, and it is conceivable that their potential infectivity would be reduced by the simple factor of dilution by a spreading factor; it is possible also that the dispersed cocci would be more effectively exposed to the defense mechanisms of the host.

The characteristic tendency in staphylococcal infections toward the formation of localized abscesses seemingly is inconsistent with the capacity of the cocci to elaborate a spreading factor. A more likely role suggested by Lack (45) is that, by depolymerizing hyaluronic acid in connective tissue, hyaluronidase may serve to remove a potential inhibitor. Work by Rogers and his associates (46) indicates that certain extracellular enzymes of the staphylococci are formed at or near the surface of the cell and are extruded in the form of a capsule. Production of the enzymes can be inhibited by certain negatively charged synthetic polymers, to which they have given the name "macroanions." These authors suggest that natural macroanions such as chondroitin sulfate, hyaluronic acid, heparin, or nucleic acids may exert a similar effect in the body. Such substances, when present in suitable concentration in a given region of the body, could thus either inhibit the formation of a factor contributing to the infectivity or virulence of the cocci, or, if it is formed, prevent its action.

Staphylococcal hyaluronidase may contribute to the severity of mixed infections with certain other bacteria or viruses. Experimental tuberculous infection in rabbits and guinea pigs has been reported to be enhanced when hyaluronidase-producing staphylococci are injected simultaneously with the tubercle bacilli (47). Similarly, mixed infections with vaccinia virus and staphylococci are considerably more diffuse than is the lesion produced by the cocci alone (48). The possible synergistic action of staphylococci and

virus is suggested by the occurrence of staphylococcal pneumonia as a complication of influenza. The question comes up whether a virus may precipitate a staphylococcal infection.

Further work on staphylococcal hyaluronidase is indicated, which might be aided by a search for inhibitors or attempts to neutralize the action of the enzyme *in vivo* by specific antihyaluronidase serum. This presupposes the eventual isolation of the enzyme in highly purified form.

## Host Factors

Closely related to the capacity of staphylococci to initiate infection is the relative susceptibility or resistance of the host. Although individuals frequently come in contact with staphylococci, and at any given point of time a relatively large proportion of persons harbor the cocci in the nasopharynx or on the skin, their mere presence is not sufficient reason for the development of infection. A common site of staphylococcal infection is the skin or subcutaneous tissue. It would seem that, apart from any toxic or other factors contributed by the cocci, local conditions at the site where they lodge may contribute significantly to the initiation of an infection. Attempts by Elek (49) to produce lesions in the skin of human volunteers led to the observation that the minimum infecting dose necessary to produce a purulent lesion was of the order of two to seven million cocci. The strains used were derived from pyogenic lesions or from the nose of carriers. With an adequate dose, a purulent lesion was produced regardless of whether the strain was toxigenic; subinfectious doses were not enhanced by plasma, toxin, or mucin. Obviously, inocula of this size would not be encountered in man's normal daily contacts with staphylococci, and the ground must be prepared to permit the establishment of a much smaller number of organisms. This would seem to occur when infection develops at the site of a foreign body such as, for example, a suture or a metal prosthetic device. In experiments reported by Elek (49), sutures carrying an estimated 30,000 cocci were inserted in the human skin and subcutaneous tissues. When the sutures were then pulled through, 80 per cent of the cocci were left in the tissue but no infection resulted. When similar sutures were allowed to remain *in situ*, severe local abscesses developed which required surgical and antibiotic treatment; it was estimated that the foreign body reaction served to enhance the infective capacity of the cocci by 500- to 10,000-fold.

It is conceivable that a condition which might delay the access of the natural cellular or humoral defenses to the site where the cocci have lodged could permit the organisms to multiply sufficiently to gain a foothold. Such a condition is realized in an area of ischemia; the local injection of a minute dose of adrenalin has been reported to immobilize the natural defenses and to enhance the infectivity of staphylococci 100-fold (50). A similar local obstruction of the blood supply as the result of a severe contusion might predispose to the establishment of an infection.

Modern methods of diagnosis or therapy employ frequent venipunctures, skin puncture, or cannulation. The development of staphylococcal cellulitis, sometimes leading to bacteremia, at the site of a skin puncture or a cannulation, has been reported by Collins and his associates (51). It appears to be possible that the local trauma caused by one of these procedures, even though it be minor, may be sufficient to provide conditions suitable for establishment of the cocci.

Whether the characteristic localization of staphylococci is entirely the result of their fixation in the tissues as a result of lymphatic blockade, as suggested by Menkin (52), is open to question. While some of the phenomena described by Menkin in his extensive studies on the inflammatory reaction have been confirmed by others (53), marked differences of opinion exist as to their interpretation (54).

When staphylococci gain entrance to the blood stream they tend to be removed rapidly by the polymorphonuclear leucocytes and by the cells of the reticulo-endothelial system. Within a few minutes after they are injected intravenously into rabbits, staphylococci disappear from the blood, a result both of the removal of circulating organisms in the splanchnic bed and of intravenous phagocytosis (28). Splanchnic removal virtually ceases when all of the circulating cocci have been ingested by the leucocytes; this is not due to exhaustion of the mechanisms of removal, for the system is fully effective when extracellular cocci again are introduced into the circulation. However, a small number of cocci may re-enter the circulation to give rise to a bacteremia of low grade. Under suitable conditions the defense mechanisms of the host may thus protect the cocci and serve to foster the infection. Staphylococci and pneumococci are removed at a constant level when introduced into the circulation experimentally by continuous infusion (55).

When injected intravenously into mice, staphylococci localize rapidly in large numbers in the spleen and liver and are found in significantly smaller numbers in the lungs and kidneys (56, 57). This is consistent with the observation by Rogers of the rapid splanchnic removal of staphylococci after intravenous injection in the rabbit. As the infection progresses, a marked rise occurs in the number of cocci in the kidneys, where abscesses eventually develop; the number in the lungs becomes stabilized at a lower level than in the kidney, and the cocci in the spleen and liver either are greatly diminished or disappear. Infection was reported to persist in mice in spite of therapy with various antibiotics administered either singly or in combination; this could not be attributed to the emergence of resistance because a large proportion of the persisters were sensitive to the antibiotics used (57).

Susceptibility to infection by staphylococci appears to be influenced by certain constitutional factors and to be markedly altered by nutritional disturbances or by certain debilitating diseases. Investigation of the factors which combine to affect susceptibility under any of these conditions provides a fertile field of study.

Although staphylococcal infections occur in persons of all ages, they are particularly common in children and young adults. Acute osteomyelitis almost characteristically occurs in the first two decades of life, while some cutaneous infections coincide with physiologic changes of puberty and adolescence.

The effect produced by disturbances of nutrition has been studied by Schaedler & Dubos (58), who found that increased susceptibility of mice to experimental infection by staphylococci and other microorganisms could be produced consistently by changes in the composition of the diet or by starvation. The change in susceptibility to infection often occurred rapidly and usually was reversible. Some studies of the effect of malnutrition on phagocytosis have been reported. In man, no significant difference was found between the phagocytosis of coagulase-positive and coagulase-negative staphylococci in severely malnourished individuals and normal controls (59). In rats, starvation for 36 hr. was reported to increase greatly the ingestion of staphylococci by leucocytes in the peritoneal fluid (60).

It would appear that host-parasite relationships may be modified by allergic reactions. Prigal & Dubos (61) have reported that many more staphylococci can be recovered from the kidneys, liver, and lungs after intravenous injection into mice sensitized to bovine serum than from control animals. The existence of a substance, probably a nucleoprotein, in extracts of cells of pathogenic staphylococci which may represent a toxic or allergenic factor is suggested by the work of Dworetzky and his associates (62). The factor is unrelated to the extracellular products of staphylococci found in culture filtrates.

## General Considerations

As was mentioned above, a large proportion of the pathogenic staphylococci that are now being encountered are resistant to one or more of the antibiotics. There is good evidence that the resistant staphylococci are represented by a relatively limited number of strains, for it is essentially a universal observation that the majority of antibiotic-resistant staphylococci fall into the broad phage Group III (63). In a recent survey of the types of staphylococci currently encountered in hospitals in the United States, it was found that the majority of hospital-acquired infections were due either to members of phage Group III or to strains of phage type 80/81, the latter having come into prominence in this country during the past three or four years (64). Antibiotic resistance may be encountered in a fair number of Group I strains, but resistant cultures of Groups II and IV are significantly less common. It was suggested by Barber & Whitehead (65) that the members of phage Group III are genetically less stable and undergo mutation to resistance more readily than those of the other groups. Another explanation is proposed by Gould (66), who reported that when penicillin-sensitive, penicillinase-negative staphylococci of Groups I or II derived from single colonies were exposed to the antibiotic on nutrient agar, two forms of penicillin-resistant colonies developed. One was nonpigmented;

the cocci produced no penicillinase, and were of the same phage type as the parent strain. The other was opaque and pigmented; the cocci produced penicillinase and were found to belong to phage Group III. If confirmed, this observation would be particularly pertinent, both to theories of the origin of resistant strains and to the present concept of the stability of phage types.

There is no convincing proof that the pathogenic staphylococci now encountered are endowed with any greater virulence than those isolated in the past. The conclusion that a strain possesses an especially high degree of virulence, based on clinical impressions of the exceptional severity of an infection (67), must necessarily be subjective and cannot, in the present state of knowledge, be confirmed by an objective test for virulence. There seems to be little doubt that the pathogenicity of staphylococci is not due primarily to any single factor but, rather, to a combination of factors (31). The number of antigen-antibody flocculation lines as determined by gel-diffusion techniques has been reported to be related to the virulence of staphylococci for mice (68).

It is frequently held, and this is admittedly subject to proof, that staphylococci derived directly from an active lesion are potentially more capable of initiating a new infection, because of their immediate past history, than those harbored by carriers. A suggestive illustration is contained in a report by Barber & Burston (69). Staphylococcal infections usually have not been regarded as occurring in epidemic form in the usual sense of that term. However, during the past three or four years outbreaks of infection have occurred of such a nature that they must be considered as being essentially epidemics. Many of these are represented by outbreaks of neonatal infection and breast abscesses of nursing mothers. At least 40 such epidemics have occurred in various parts of the world, including 19 in the United States (70); they have given rise to the recommendation that neonatal staphylococcal infections and breast abscesses in nursing mothers should be made reportable diseases (70, 71). Outbreaks of this nature suggest a high communicability of the staphylococci and an infectivity that has been enhanced as a result of selection.

## LITERATURE CITED

1. Pillemer, L., and Robbins, K. C., *Ann. Rev. Microbiol.*, **3**, 265-88 (1949)
2. Kleiger, B., Blair, J. E., and Hallman, F. A., *Arch. Surg.*, **45**, 571–77 (1942)
3. Kellaway, C. H., MacCallum, P., and Tebbutt, A. H., *Med. J. Australia*, **2**, 2–30, 38–52, 58–64 (1928)
4. Kleiger, B., and Blair, J. E., *Surg. Gynecol. Obstet.*, **71**, 770–77 (1940)
5. Kleiger, B., and Blair, J. E., *Arch. Surg.*, **46**, 548–54 (1943)
6. Marks, J., *J. Pathol. Bacteriol.*, **64**, 175–86 (1952)
7. Elek, S. D., *Ann. N. Y. Acad. Sci.*, **65**, 85–89 (1956)
8. Glenny, A. T., and Stevens, M. F., *J. Pathol. Bacteriol.*, **40**, 361–77 (1938)
9. Smith, M. L., and Price, S. A., *J. Pathol. Bacteriol.*, **59**, 69–78 (1947)
10. Williams, R. E. O., and Harper, G. J., *J. Pathol. Bacteriol.*, **59**, 69–78 (1947)

11. Blair, J. E., *Bacteriol. Rev.*, **3**, 97–146 (1939)
12. Elek, S. D., and Levy, E., *J. Pathol. Bacteriol.*, **62**, 541–54 (1950)
13. Topley, W. W. C., and Wilson, G. S., *Principles of Bacteriology and Immunity*, 4th ed., 1149–52 (Williams and Wilkins Company, Baltimore, Md., 2331 pp., 1955)
14. Surgalla, M. J., Bergdoll, M. S., and Dack, G. M., *J. Lab. Clin. Med.*, **41**, 782–88 (1953)
15. Bergdoll, M. S., *Ann. N. Y. Acad. Sci.*, **65**, 139–43 (1956)
16. Thatcher, F. S., Matheson, B. H., and Simon, W. R., *Can. J. Microbiol.*, **1**, 401–11 (1955)
17. Surgalla, M. J., Bergdoll, M. S., and Dack, G. M., *J. Immunol.*, **72**, 398–403 (1954)
18. Levi, L., Matheson, B. H., and Thatcher, F. S., *Science*, **123**, 64–65 (1956)
19. Dack, G. M., *Food Poisoning*, 3rd ed., 152–54 (University of Chicago Press, Chicago, Ill., 251 pp., 1956)
20. Valentine, F. C. O., *Lancet*, **II**, 526–31 (1936)
21. Jackson, A. W., and Little, R. M., *Can. J. Microbiol.*, **3**, 101–02 (1957)
22. Rogers, D. E., and Tompsett, R., *J. Exptl. Med.*, **95**, 209–30 (1952)
23. Lyons, C., *Brit. J. Exptl. Pathol.*, **48**, 411–22 (1937)
24. Tager, M., *Yale J. Biol. and Med.*, **20**, 369–80 (1948)
25. Smith, W., and Hale, J. H., *Brit. J. Exptl. Pathol.*, **25**, 101–10 (1944)
26. Smith, W., Hale, J. H., and Smith, M. M., *Brit. J. Exptl. Pathol.*, **35**, 57–67 (1947)
27. Hale, J. H., and Smith, W., *Brit. J. Exptl. Pathol.*, **26**, 209–16 (1945)
28. Rogers, D. E., *Ann. N. Y. Acad. Sci.*, **65**, 73–84 (1956)
29. Menkin, V., and Walston, H. D., *Proc. Soc. Exptl. Biol. Med.*, **32**, 1259–63 (1935)
30. Fisher, A. M., *Bull. Johns Hopkins Hosp.*, **59**, 393–414 (1936)
31. Lack, C. H., and Wailling, D. G., *J. Pathol. Bacteriol.*, **68**, 431–43 (1954)
32. Spink, W. W., and Vivino, J. J., *J. Clin. Invest.*, **21**, 352–56 (1942)
33. Ekstedt, R. D., *J. Bacteriol.*, **72**, 157–61 (1956)
34. Rammelkamp, C. H., Jr., and Lebovitz, J. L., *Ann. N. Y. Acad. Sci.*, **65**, 144–51 (1956)
35. Tager, M., and Lodge, A. L., *J. Exptl. Med.*, **94**, 73–85 (1951)
36. Duthie, E. S., and Lorenz, L. L., *J. Gen. Microbiol.*, **6**, 95–107 (1952)
37. Rammelkamp, C. H., Jr., Hezebicks, M. M., and Dingle, J. H., *J. Exptl. Med.*, **91**, 295–307 (1950)
38. Duthie, E. S., *J. Gen. Microbiol.*, **7**, 320–26 (1952)
39. Tager, M., *Ann. N. Y. Acad. Sci.*, **65**, 109–18 (1956)
40. Tager, M., and Hales, N. B., *J. Immunol.*, **60**, 1–9 (1948)
41. Fusillo, M. H., and Jaffurs, W. J., *J. Bacteriol.*, **70**, 481 (1955)
42. Fisher, A. M., *Bull. Johns Hopkins Hosp.*, **59**, 412–26 (1936)
43. Lack, C. H., *Nature*, **161**, 559–60 (1948)
44. Cruikshank, R., *J. Pathol. Bacteriol.*, **45**, 295–303 (1937)
45. Lack, C. H., *Ann. N. Y. Acad. Sci.*, **65**, 103–08 (1956)
46. Rogers, H. J., *Ann. N. Y. Acad. Sci.*, **65**, 132–38 (1956)
47. Bergqvist, S., and Packalén, T., *Acta Tuberc. Scand.*, **23**, 250–60 (1949)
48. Lack, C. H., *Brit. J. Exptl. Pathol.*, **29**, 191–202 (1948)
49. Elek, S. D., *Ann. N. Y. Acad. Sci.*, **65**, 85–90 (1956)

50. Evans, D. G., Miles, A. A., and Niven, J. S. F., *Brit. J. Exptl. Pathol.*, **29**, 20–39 (1948)
51. Collins, H. S., Helper, A., Blevins, A., and Olenberg, G., *Ann. N. Y. Acad. Sci.*, **65**, 222–34 (1956)
52. Menkin, V., *J. Exptl. Med.*, **64**, 485–502 (1936); *Am. J. Pathol.*, **16**, 13–22 (1940); *Arch. Pathol.*, **36**, 269–88 (1943)
53. Duthie, E. S., and Chain, E., *Brit. J. Exptl. Pathol.*, **20**, 417–29 (1939)
54. Miles, A. A., and Miles, E. M., *Brit. J. Exptl. Pathol.*, **24**, 95–107 (1943)
55. Martin, S. P., and Kerby, G. P., *J. Exptl. Med.*, **92**, 45–49 (1950)
56. Smith, J. M., *Ann. N. Y. Acad. Sci.*, **65**, 67–72 (1956)
57. McCune, R. M., Jr., Dineen, P. A. P., and Batten, J. C., *Ann. N. Y. Acad. Sci.*, **65**, 91–102 (1956)
58. Schaedler, R. W., and Dubos, R. J., *J. Exptl. Med.*, **104**, 67–84 (1956)
59. Balch, H. W., and Spencer, M. J., *J. Clin. Invest.*, **33**, 1321–28 (1954)
60. Kuna, A., Blattberg, B., and Reiman, J., *Proc. Soc. Exptl. Biol. Med.*, **77**, 510–14 (1954)
61. Prigal, S. J., and Dubos, R. J., *Proc. Soc. Exptl. Biol. Med.*, **93**, 340–43 (1956)
62. Dworetezky, M., Baldwin, H. S., and Smart, K. M., *J. Allergy*, **27**, 39–49 (1956)
63. Blair, J. E., *Ann. N. Y. Acad. Sci.*, **65**, 152–60 (1956)
64. Blair, J. E., and Carr, M., *J. Am. Med. Assoc.*, **166**, 1192–96 (1958)
65. Barber, M., and Whitehead, J. E. M., *Brit. Med. J.*, **11**, 565–69 (1949)
66. Gould, J. C., *Nature*, **176**, 176 (1955)
67. Rountree, P. M., and Freeman, B. M., *Med. J. Australia*, **42**, 157–61 (1955)
68. Howard, J. G., *J. Pathol. Bacteriol.*, **68**, 177–86 (1954)
69. Barber, M., and Burston, J., *Lancet*, II, 578–83 (1953)
70. Shaffer, T. E., Sylvester, R. F., Baldwin, J. N., and Rheins, M. S., *Am. J. Public Health*, **47**, 990–94 (1957)
71. Ravenholt, R. J., and LaVeck, G. D., *Am. J. Public Health*, **46**, 1287–96 (1956)

# MICROBIOLOGICAL ASPECTS OF RADIATION PRESERVATION OF FOOD[1]

By C. F. Niven, Jr.
*American Meat Institute Foundation, The University of Chicago, Chicago, Illinois*

## Introduction

The idea of preserving foods by subjecting them to ionizing radiations is not new. The lethal effect of x-rays and radioactive isotopes has been realized since their discovery and isolation. In 1929, a patent was issued in France which proposed the use of high energy radiation for processing foods.

Investigations concerning the use of ionizing radiation for food sterilization and processing gained impetus immediately following World War II because of the increased availability of radioactive isotopes, as well as improved technologic developments in ion-acceleration devices. This "unique" principle of food preservation naturally possessed an appeal because it promised to be the first new concept in this field since the inception of thermal processing.

Many foods either cannot be preserved by heat, or are greatly damaged or altered with regard to their acceptability as a result of thermal processing. To sterilize such foods by some other method would be desirable. The term "cold sterilization" has therefore evolved, especially in the popular literature, to denote the processing of foods with ionizing radiations.

Radiation processing of foods has received widespread publicity in the lay press, and generally has been exaggerated beyond objectivity with regard to its implications. Even among the professional investigators engaged in this field of endeavor rather wide variance of opinion appears to exist as to the future application of ionizing radiation in the food industry. That all foods can be sterilized by radiation cannot be denied. However, most foods subjected to sterilizing doses are altered to some extent with regards to flavor, odor, color, or texture. Flavor and odor changes can only be measured subjectively. Herein lies the basis for disagreement among the investigators concerning the future potentialities of radiation processing of foods. Unfortunately, the only known criterion for resolving these differences in opinion is the ultimate widespread acceptance or rejection by the consumer of such processed foods.

Current interest in the radiation processing of foods was stimulated shortly after World War II by the work of Proctor, Goldblith, and co-

[1] The survey of the literature pertaining to this review was concluded in January, 1958.

workers at the Massachusetts Institute of Technology, and by Brasch & Huber (1). Foreseeing the accumulation and availability of rather large quantities of radioactive fission waste materials, the United States Atomic Energy Commission encouraged investigations along these lines by sponsoring research grants in a number of institutions. The Quartermaster Food & Container Institute for the Armed Forces realized the vast potentialities of radiation processing of military rations as an effective method of preservation, and, therefore, initiated the sponsorship of a well-coordinated program of research in a large number of research laboratories in 1954. This Quartermaster Institute program is continuing, and has progressed to the point of initiating the construction of a pilot plant near Stockton, California, for the purpose of testing the feasibility of processing foods on a rather large-scale basis.

As the title infers, this review is not intended to discuss in detail the theoretic aspects of the lethal action of ionizing radiations on microorganisms. Several such reviews have been published, and the reader is referred to Kelner *et al.* (2) for a comprehensive summary of the radiation effects upon the bacterial constituents and the mutagenic properties of radiation; and to Kimball (3) for the nongenetic effects of radiation on microorganisms. Such reviews should prove to be of value to the food microbiologist in gaining an appreciation of the basic concepts of the biologic action of ionizing radiations. No attempt will be made to review the voluminous literature concerning the technologic aspects of irradiation, nutritional studies, safety aspects, or chemical studies on irradiated foods as related to the production of undesirable flavors and odors, texture changes, or discolorations. The need for a novel preservation method is greatest among the more expensive, high-protein foods, such as meats and dairy products. Unfortunately, these foods appear to be most sensitive to radiation damage with regards to the production of undesirable odors, flavors, and discolorations (8).

Appropriate reviews on the general area of the microbiologic aspects of radiation preservation of foods have been published by Hannan (4, 5), Hannan & Thornley (6), and Bellamy *et al.* (7). The reader is referred to these publications for a broad appreciation of the general problems which are encountered in the preservation of foods by ionizing radiations.

## Radiation Sources for Food Processing

Among the different types of ionizing radiations there are only two that seem to have applicability in processing foods. X-rays or $\gamma$-rays are electromagnetic radiations having high penetrative capacity. Therefore, the thickness of the food sample is, in most instances, not of major concern when this type of radiation is employed. Cathode or $\beta$-rays are streams of high-speed electrons that have relatively low penetrative efficiency. When they

are employed the thickness of the sample becomes of major concern. The effective depth of penetration of β-rays depends upon their energy level. For example, at an energy level of one million electron volts (Mev) the effective depth of penetration is approximately 0.25 cm. in a substance of unit density, or 0.75 cm. when irradiated from opposite sides (6, 9). At the 10 Mev-level the effective penetration depth in increased to 3.8 cm., or 8.6 cm. when irradiated from opposite sides. In contrast, the effective depth of penetration of γ-rays from the radioactive isotope, $Co^{60}$, is 10 cm. on one side, or 40 cm. when irradiated from all sides.

Common sources of cathode rays for both investigational and commercial use are the Van de Graaff electrostatic generator (10), the resonant transformer (11), and the linear accelerator (12). The linear accelerator is receiving current interest because of the ease with which cathode rays with very high energy levels can be attained, thus achieving effective depth of penetration. Some concern is expressed with regard to the upper limit of the energy level of cathode rays that can be employed without inducing temporary radioactivity among some of the food constituents or the container. Hannan & Thornley (6) believe that the upper limit for safety is aproximately 10 Mev.

Gamma-ray sources for food processing may be obtained from a variety of radioactive isotopes, including gross-waste fission products from atomic energy development (13), radioactive by-products of commercial reactors (14), as well as spent fuel rods. Cobalt-60 has become an excellent and convenient source of γ-radiation for research purposes. For commercial use in the food industry, $Cs^{137}$ is particularly attractive because of its relatively long half-life (over 30 years).

Other than by conventional methods, x-rays can be generated by the insertion of a suitable target in the electron beam of the cathode-ray generators described above. However, at the lower energy levels, a sizable loss in efficiency is experienced. Soft x-rays are useful for research purposes, but their low penetrative capacity has detracted from their potential usefulness for food processing.

Alpha particles (helium nuclei) are lethal to bacteria, but they have an extremely low penetrative capacity. Neutrons are lethal and have effective penetrative capacity, but their ability to induce radioactivity in the irradiated material eliminates their consideration for the processing of foods.

The unit of measurement for recording the dose of high energy radiation to which a material is subjected has been the rep (roentgen equivalent physical). A rep generally has been defined as the absorption of 93 ergs of energy per gram of substance having unit density. However, because of some confusion with regard to the quantification of the rep, the term "rad" is now gaining widespread acceptance. A rad is arbitrarily defined as the absorption of 100 ergs of energy per gram of substance having unit density.

## Mechanism of Action

Regardless of the type of radiation, the end bactericidal effectiveness appears to be the same. Goldblith *et al.* (15) reported that under controlled conditions, high-energy cathode rays, x-rays from a particle accelerator, and $\gamma$-rays from a $Co^{60}$ source had essentially the same lethal effectiveness. It is difficult to obtain accurate quantitative data of this type because of the inherent difficulties in dosimetry and maintaining uniformity of the radiation dose throughout the sample.

The dose rate to which bacteria are subjected appears to make little difference upon their survival. Bellamy & Lawton (16) reported that the survival of *Escherichia coli* was the same when the dose rate was varied over a rather wide range.

The energy from ionizing radiations is dissipated in the food primarily by the release of electrons from the atoms through which it passes. As a result, a positively charged ion is generated. The ejected electron combines with another atom to form a negatively charged particle. A large fraction of food material or of a bacterial cell is water. Therefore, the formation of these highly reactive ion pairs, or free radicals, from water becomes of importance.

If an ionization takes place within a vital molecule of the cell, with a resulting lethal action, the effect is called a direct one. Thus, the direct target theory of Lea (17) has evolved with regard to the mechanism of action of ionizing radiations. If the ionization occurs in some constituent of the solvent in the immediate vicinity of the vital center of the cell and the ionized particles chemically inactivate the vital center, the effect is called an indirect one.

It appears as though both theories must be taken into consideration in explaining the mechanism of the lethal action of radiations. Regardless of which mechanism is predominant in a given case, the magnitude of migration of the radicals formed seems to be of a small order (18).

Bacteriologists are somewhat hesitant to refer to bacteria as being "killed" by radiation when they fail to produce macrocolonies on suitable plating media. The term "inactivation" is more commonly employed. This term would appear to be a more appropriate one in the light of the continued metabolic activities of the irradiated cells. Gunter (19) noted that nonsurviving yeast cells, as well as some bacterial cells, divided at least once after x-ray treatment. The formation of enlarged cells among the nonsurvivors was observed with every strain tested. Burns (20) reported that when mature diploid yeast cells were irradiated with small doses of x-ray just before division, the second division after irradiation was greatly delayed. The third and subsequent divisions were not delayed. The results were interpreted to mean that x-rays damaged the same cell organelles responsible for the lethal effects of radiation, but the damage was qualitatively different from that which results in inactivation.

The survivor curve of microorganisms subjected to increasing doses of radiation is generally considered to yield a straight line when the logarithm of survivors is plotted against dose. However, rather complex survivor curves frequently can be encountered, as demonstrated by Gunter & Kohn (21). These authors also demonstrated the relatively high radiation resistance of budding yeast cells. A lag period of varying proportions before logarithmic inactivation occurs is commonly observed when microorganisms are irradiated in foods or complex media. Such results cannot be explained wholly on the basis that the cells occur in clumps or are multinucleated.

## Factors Affecting Radiation Resistance of Microorganisms

Several reviews have been published concerning the environment of bacterial cultures during irradiation as affecting their radiation resistance. The reader is referred to Kelner *et al.* (2), and to Hollaender & Stapleton (22) for more detailed discussion of this subject.

One of the more important factors affecting the radiation resistance of bacteria is the presence of oxygen during irradiation. Complete removal of oxygen from the cell suspension may increase the radiation resistance of *E. coli* cells up to threefold. Other environmental changes, prior to or during irradiation, result in increased resistance, presumably as different manifestations of oxygen depletion. The addition of an oxidizable substrate prior to irradiation is one such example. The addition of reducing substances to the suspending medium, such as hydrosulfite, dimercaptopropanol, β-mercaptoethylamine (23, 24), and other sulfhydryl compounds may result in a dramatic increase in radiation resistance.

An exception to the general rule of the effect of oxygen removal was observed with *Pseudomonas geniculata,* a highly aerobic, psychrophilic organism causing spoilage of fresh meat (25). Reduced oxygen tension did not alter the radiation resistance of this species. On the other hand, population levels in the order of $8 \times 10^6$ cells per ml. required a threefold greater dose of γ-radiation for a 99 per cent inactivation than that required at initial population levels of $10^6$ cells per ml. or less. High population levels of a *Micrococcus* sp. did not alter the radiation resistance of low numbers of the *Pseudomonas* cultures.

The removal of water by freezing or drying generally affords a protective effect against radiation damage. It is presumed that radiation of dried cells lowers the production of ionized particles and retards the migration of those that are produced. Wood & Taylor (26) reported that yeast strains show an increasing radiation resistance when irradiated under anoxic conditions, in the frozen state, and a combination of the two. They concluded that only about one-third of the lethal effect could be accounted for by the direct action of the radiation. Stapleton & Eddington (27) observed that oxygen-saturated suspensions of *E. coli* showed a significant decrease in

sensitivity at subfreezing temperatures and a discontinuous change at the freezing point. In the absence of oxygen, however, no significant change in sensitivity occurred with decreasing temperatures below the freezing point. These findings are consistent with the hypothesis that subfreezing temperatures protect against inactivation of bacteria by interference with the production of toxic agents, as well as their migration to the sensitive sites within the cell. Proctor *et al.* (28) reported that *Bacillus subtilis* spores were more resistant to high energy cathode-rays under anoxic conditions or when lyophilized. However, frozen suspensions were more sensitive to radiation than nonfrozen suspensions.

A more recent publication by Proctor *et al.* (29) indicated that when *Bacillus thermoacidurans* spores suspended in a phosphate-citrate buffer were irradiated, no difference in resistance could be detected in the presence of oxygen, air, or nitrogen, whether frozen or nonfrozen. In a broth suspension, moderate differences in the radiation resistance of the spore suspension could be detected under the different environmental conditions. The spores were more sensitive at lower pH values, and also tended to become more sensitive as the spores were aged in the refrigerator.

√ Temperature variations above the freezing point are not considered to affect markedly the radiation sensitivity of microorganisms. However, a striking exception to this rule has been observed by Kempe (30). Spores of *Clostridium botulinum* were found to be most sensitive over a narrow range at about room temperature. In contrast, spores of a putrefactive anaerobe, PA3679, were most sensitive at 85°C. These results need confirmation in the light of their implications with regards to food processing.

√ Hydrogen peroxide is known to be synthesized and to accumulate when water is subjected to ionizing radiations. In accordance with this fact, Williams & Kempe (31) reported that catalase in the suspending medium significantly reduced the lethal action of $\gamma$-rays for *C. botulinum* and PA3679 spores. This finding may become important in evaluating effective sterilizing doses of radiation for foods rich in catalase.

The stage in the growth cycle of bacteria as they are irradiated appears to influence their radiation resistance. *E. coli* is most resistant in the lag phase just prior to active cell division (2). An active decay in resistance occurs as the cells proceed through the log phase, reaching a minimum at the end of the log phase. The type of plating medium employed in determining the number of "survivors" may exert a profound influence upon the apparent lethal effects of radiation (32). Also, incubation of the plates at temperatures below that which is optimal results in a partial alleviation of the apparent effects of x-rays on *E. coli* (2).

Thus, environment, before, during, and after irradiation, may exert a profound effect upon the radiation resistance of a microorganism. It is difficult to reconcile some of these observations with the direct target theory

of Lea. Microorganisms are usually much more resistant when irradiated in a food as compared to a buffered suspension. For example, *P. geniculata* is considerably more resistant in meat than in broth (25), *B. subtilis* spores are more resistant in pea purée (28) and *B. thermoacidurans* spores are more resistant in tomato juice than in buffered suspensions (29). Therefore, for intelligent application of the results for food processing, the potential spoilage microorganisms should be irradiated in the food in question. The protective substances in food probably are of a multiple variety that exist principally in the water-soluble fraction.

Just as some chemicals increase the radiation resistance of microorganisms, some serve as so-called free radical acceptors (33) to aid in protecting the foods from severe radiation damage. Examples of these food protectors are *iso*-ascorbic and ascorbic acids, sulfite, fumarate, and glutamate. Some of these free radical acceptors may protect the contaminating bacteria, in which case no advantage is gained in their addition to the food. The intensity of radiation-induced flavors and odors of foods also may be lessened by irradiating in the frozen state, by controlling the oxygen content, or by holding the irradiated food at room temperature for a period of time (34).

## Relative Radiation Resistance of Microorganisms

Several expressions have been employed by different investigators to denote the quantitative inactivation of microorganisms by ionizing radiations. Mean lethal dose ($D_0$) from Lea's target theory expression is often employed, which is the dose required to effect a 37 per cent survival. This expression is useful when a straight-line relationship exists between the logarithm of survivors and the dose. The radiation dose required to reduce the bacterial population by two ($LD_{99}$) or more decimal values is often used. The dose required for total sterilization is frequently recorded, but such figures are difficult to evaluate, especially when initial populations are not stated.

The frequent occurrence of diverse types of survivor curves, particularly one showing an initial lag in inactivation, complicates quantitative expressions of radiation resistance. The variable resistance of a given mircoorganism under different environmental conditions also needs to be taken into account. In spite of these difficulties some generalizations can be made.

Bacterial endospores are usually highly resistant under any conditions tested. Just as in thermal sterilization of foods, the inactivation of endospores is the most important consideration in radiation sterilization. Other than high resistance of the endospores, there seems to be no real correlation between radiation resistance and thermal resistance among bacteria. There appears to be no method of predicting the radiation resistance of a microorganism by having knowledge of its metabolic capacities. The most im-

portant criterion is the species to which the strain in question belongs. Even within one species, however, rather wide differences in radiation resistance are sometimes encountered under apparently identical environmental conditions.

Gram-positive bacteria are generally more resistant to radiation than Gram-negative bacteria (35, 36). When fresh meat is irradiated with 100,000 rads, the surviving microflora may be changed from predominantly Gram-negative to Gram-positive.

Table I presents some examples of the radiation resistance of some potential food-spoilage microorganisms. The recorded $LD_{99}$ doses serve only to give a rough comparison of the resistances among widely different microorganisms, and to demonstrate ranges of resistance among strains of a given species tested under the same conditions.

In a series of publications, Chandler *et al.* (36, 43, 44) employed a novel

TABLE I

EXAMPLES OF RADIATION RESISTANCE AMONG SOME POTENTIAL FOOD-SPOILAGE MICROORGANISMS

| Species | Dose for 99% Inactivation (rads×10³) | References |
|---|---|---|
| Spores[a] | | |
| *Clostridium botulinum* | 800–1300 | (31, 37) |
| *Clostridium sporogenes*, PA3679 | 800 | (37) |
| *Thermophilic anaerobe*, 3814 | 500 | (37) |
| *Bacillus stearothermophilus*, 1518 | 600 | (37) |
| Vegetative Cells[b] | | |
| *Pseudomonas geniculata* | 7–23 | (25) |
| *Lactobacillus* (homofermentative) | 165–220 | (38) |
| *Lactobacillus* (heterofermentative) | 55–80 | (38) |
| *Leuconostoc* sp. | 40–113 | (39) |
| *Streptococcus faecalis* | 81–236 | (40) |
| *Streptococcus faecium* | 174–654 | (40) |
| *Microbacterium thermosphactum* | 61–89 | (39) |
| *Staphylococcus aureus* | 44 | (39) |
| *Micrococcus* sp. | 67–200 | (40) |
| *Micrococcus rubens* | 460 | (41) |
| *Micrococcus roseus* | 1400–2000 | (42, 41) |
| *Debaryomyces kloeckeri* | 165–200 | (40) |
| *Torulopsis candida* | 61–118 | (40) |
| *Candida* sp. | 50–250 | (40) |

[a] Suspended in phosphate buffer.
[b] Suspended in broth.

technique of impregnating small filter discs with 0.1 ml. of cultures representing a wide variety of microorganisms, including nonsporeforming bacteria, bacterial endospores, and yeasts and molds. The discs were enclosed in films and the minimum cathode ray dose necessary to sterilize the discs was determined. Although the results are at most semiquantitative, they serve to give a rough estimate of the radiation resistance of the large number of microorganisms employed. Gram-positive bacteria were generally more resistant than Gram-negative bacteria. Yeasts were more resistant than molds. Some *Candida* strains were found to have resistance comparable to that of bacterial endospores.

Of profound interest to the food microbiologist is the finding by Anderson *et al.* (42) of a *Micrococcus* which is very resistant to radiation. This organism, which resembles *M. roseus* or *M. rubens tetragenus,* was isolated repeatedly from ground beef and pork following $\gamma$-irradiation doses of 2-3 $\times$ $10^6$ rads. Pure cultures on agar slants survived 6 $\times$ $10^6$ rads. This order of resistance places these cultures as the most radiation-resistant of all bacteria yet detected in spite of the fact that they do not form spores.

Anderson *et al.* reported that the radiation-resistant *Micrococcus* is Gram-positive, red-pigmented, facultative, strongly catalase-positive, unable to reduce nitrate, and very sensitive to salt. Growth is largely inhibited by concentrations of sodium chloride above two per cent. Morphologically, the microorganism has a peculiar appearance in that the rather large cocci form dense tetrad packets which make it difficult to distinguish the individual cells by ordinary staining methods.

Berk (41) extended the studies on the radiation-resistant micrococci of Anderson *et al.* and noted that a $\gamma$-ray dose of 1,400,000 to 2,000,000 rads was necessary to effect a 99 per cent inactivation. A characteristically long lag was consistently observed in the survivor curve before a logarithmic inactivation occurred. This type of response might be expected in an organism that exists in packets.

Berk noted that the radiation-resistant *Micrococcus* was highly aerobic. Little or no growth could be achieved under anaerobic conditions or at 37°C. It failed to ferment or oxidize glucose as an energy source and appeared to obtain energy for growth by oxidizing amino acids. The cultures were very sensitive to heat and did not survive more than a few minutes at 60°C.

Prompted by these observations, Anderson *et al.* (42), as well as Berk (41), determined the radiation resistance of other red-pigmented micrococci and noted that they also were radiation-resistant (Table I). However, their resistance was less than half of that observed for the micrococci from meat. These results would tend to relate the radiation resistance to the red pigment. Berk was unable to isolate a nonpigmented mutant from the parent culture, but the cultures could be grown under reduced oxygen tension with

the production of no visible pigment. These nonpigmented cultures were just as resistant to radiation as the cultures containing pigment.

The existence of such microorganisms as the radiation-resistant *Micrococcus* certainly must be kept in mind for any contemplated radiation processing of a food which contains a low salt content and which receives no heat treatment.

## Required Sterilization Doses for Foods

Until recently most of the chemical investigations concerning the prevention of radiation-induced damage to foods proceeded on the basis that approximately $2 \times 10^6$ rads constituted an effective sterilizing dose for foods. The origin of this assumption is rather obscure, but more recent work indicates that a safe sterilization will require considerably higher doses.

In the conventional thermal processing of foods, spores of certain thermophilic anaerobes and aerobes, as well as the nontoxigenic putrefactive anaerobe, PA3679, are found to be the most heat-resistant and most commonly encountered in food spoilages due to marginal processing. In contrast to this picture, Morgan & Reed (37) noted that the spores of *Clostridium botulinum* were the most radiation-resistant among the spore suspensions tested. Kempe (30) reported that a dose of $3.7 \times 10^6$ rads was necessary to sterilize meat that had been inoculated with 40,000 *C. botulinum* spores per gram.

The food processor has followed the classical studies of Esty & Meyer on the thermal resistance of *C. botulinum* spores to calculate safe thermal processing levels of foods. These studies indicated that a heat processing equivalent to 2.78 min. at 121°C. was required to reduce $1 \times 10^{12}$ spores to less than one surviving spore. By employing the best available data pertaining to survivor curves of *C. botulinum* subjected to high energy radiation, Schmidt (45) has calculated that a comparable radiation processing to effect the same degree of safety would require a minimum of $4.5 \times 10^6$ rads. These calculations appear to be entirely reasonable, especially in the light of the fact that *C. botulinum* spores are among the most radiation-resistant spores. The inference exists that the hazard of food spoilage due to *C. botulinum* would be greater in marginally radiation-processed foods than in heat processed foods. Such severe radiation doses will complicate even further the problem of preventing radiation-induced damage to foods.

## Complementary Effects of Radiation and Heat

Morgan & Reed (37) noted that the thermal resistance of spore suspensions of strain PA3679 was significantly reduced if they were first subjected to a sublethal dose of $\gamma$-radiation. Kempe (46) confirmed this phenomenon using spore suspensions of *C. botulinum*. The heat sterilizing value of the spore suspension could be reduced up to fourfold by preirradiation

in phosphate buffer. The effect was less pronounced when the spores were suspended in gelatin and nutrient broth. Increased amounts of γ-radiation caused increased degrees of heat sensitization. Conversely, preliminary heat shocking at 99°C. did not affect the lethal action of subsequent γ-irradiation.

Kempe, Graikoski, & Bonventre (47) extended this work by testing *C. botulinum* spores in ground beef. In this instance, the effect of preirradiation was not nearly so dramatic. Approximately one million rads of γ-radiation were required to reduce significantly the thermal processing needed for sterility.

Kan, Goldblith, & Proctor (48) further confirmed the effect of preirradiation on the thermal resistance of spores of PA3679 and *Bacillus cereus*. The effect could be demonstrated in ham menstra. Again, radiation sensitivity was not affected by prior heating of the spores.

The mechanism of the sensitizing of spores to heat by preirradiation has not been elucidated. However, it is interesting to recall that Clark (49) presented evidence which indicated that radiation effected a sensitization of proteins to heat denaturation.

Although these observations are of theoretical interest, it is doubtful whether they will have commercial application in the light of the double processing that would be required, both of which would be reasonably severe. If a food displayed advantages from an acceptability standpoint with the double processing, the technique might be useful in some military rations.

Even if radiation sterilization of some foods becomes feasible, at least a light heat processing either before or after radiation may be necessary to inactivate deteriorative enzymes in the food, or else the food will have to be maintained under refrigeration. Enzymes, especially in foods, are known to be very resistant to ionizing radiations. Kirn, Urbain, & Czarnecki (50) discuss the difficulties of maintaining quality in radiation-sterilized foods, especially meats, when stored at room temperature for extended periods of time.

### Radiation Pasteurization of Foods

Several laboratories have actively investigated the feasibility of extending the refrigerated life of foods by subjecting them to relatively low doses of ionizing radiation while holding radiation damage to a minimum. Two approaches have been employed—namely, irradiating at a level of 200,000 rads or less to inactivate the more important spoilage microorganisms, and irradiating at levels up to $10^6$ rads to inactivate essentially all microorganisms except endospores. The sensitivity of the food toward radiation damage determines the level to be employed.

The need for radiation pasteurization is greatest among those foods that cannot be pasteurized by conventional methods or preserved in some effective manner. Fresh red meats are good examples.

Niven (38), Wolin, Evans & Niven (25), and Schweigert *et al.* (51) have demonstrated the effectiveness of subjecting prepackaged fresh beef to $10^5$ rads or less in order to achieve an extension of refrigerated shelf life. By this method it would be possible to prepackage fresh meats at the wholesale level, a practice not hitherto possible because of the rather high perishability of the food.

When prepackaged fresh meats are held at refrigerator temperatures, spoilage becomes evident within a few days because of the rapid growth of psychrophilic pseudomonads, principally *Pseudomonas geniculata* (25). A $\gamma$-ray dose of $10^5$ rads or less will completely inactivate all pseudomonads on the meat. If such treated meat is held at approximately 2°C. the life is extended significantly, but it will eventually become spoiled because of the growth of surviving microorganisms. The most important surviving bacterium has proved to be identical with *Microbacterium thermosphactum,* as described by McLean & Sulzbacher (52). Thus, a unique type of spoilage is encountered in the prepackaged meat.

Drake, Evans & Niven (39) reported that the predominant spoilage flora of cured meats consisted of *M. thermosphactum,* various types of lactic acid bacteria, micrococci, and yeasts. These microorganisms were found to be much more radiation-resistant than the fresh-meat pseudomonads. Therefore, a significant extension of the refrigerated life of cured meats was difficult to achieve unless subjected to at least $4 \times 10^5$ rads. At this dose level radiation-induced surface discoloration of the meat was encountered. Cured meats inherently are less perishable than fresh meats because of the thermal processing to which they have been subjected and because of the partial preservative action of the curing agents.

Thornley (53) achieved a dramatic increase in the refrigerated life of minced chicken meat held anaerobically by subjecting it to 250,000 rads of $\gamma$-radiation. Some of the irradiated samples were wholesome after 80 days. The eventual spoilage microorganisms in the irradiated samples were *M. thermosphactum* and *Streptococcus faecium.*

Nickerson *et al.* (54) were able to increase significantly the refrigerated life of haddock fillets by irradiating with $4\text{-}7 \times 10^5$ rads from a cathode ray source.

Effective refrigerated preservation of milk has been achieved with low levels of radiation by Gaden, Henley & Collins (55), and by Nickerson *et al.* (56). A dose of $5 \times 10^5$ rads reduced radically the viable population in raw milk.

Niven & Chesbro (35, 57) demonstrated a complementary effect of low levels of $\gamma$-radiation and the tetracycline antibiotics for the preservation of prepackaged fresh meats. A dose of 90,000 rads completely inactivated the psychrophilic, Gram-negative flora in the meat, but was less effective on the Gram-positive bacteria. Appropriate concentrations of tetracycline anti-

biotics were effective in retarding the growth of the surviving Gram-positive bacteria. With a combination of irradiation and antibiotics the refrigerated life of the meat was very effectively increased. Yeasts eventually spoiled the meat treated in this manner. The addition of sorbic acid to prevent yeast growth made it possible to preserve the refrigerated fresh meat to a point where nonmicrobial changes became predominant in limiting the shelf life.

## Public Health Aspects

The general public health aspects of irradiated foods, including possible alterations of nutritive value and wholesomeness, have been reviewed by Nickerson, Proctor & Goldblith (56). These areas will not be discussed in this review.

The question is often asked as to whether irradiation of a food, especially with sublethal doses, may not alter the flora in such a manner as to favor the growth of certain harmful microorganisms. This question can only be answered by considering individually the foods to be so treated. If radiation pasteurization of milk is to be considered as a substitute for conventional methods, several complications may arise. Milk is pasteurized not only to extend its refrigerated life but to kill human pathogens that may be present. The minimum processing schedule for the conventional pasteurization of milk, historically, was based primarily upon the thermal resistance of *Mycobacterium tuberculosis*. Little is yet known concerning the radiation resistance of this pathogen or several other pathogens that may inhabit raw milk.

In contrast to their thermal resistance, the viruses are known to be very radiation-resistant. Jordon & Kempe (58) found that a dose of $3.6 \times 10^6$ rads was necessary for complete sterilization of the Lansing polio, St. Louis encephalitis, and Western equine encephalomyelitis viruses when an initial population of one million $LD_{50}$ doses was used. The vaccinia virus was somewhat less resistant. The viruses were more resistant in whole brain suspensions than when partially purified.

The phosphatase enzyme of milk is not destroyed even with radiation doses that are 10 times those required for the sterilization of raw milk (59). Therefore, new criteria would have to be sought for determining the adequacy of radiation pasteurization of milk. In the light of these difficulties it appears very unlikely that radiation will become competitive with conventional processing methods for dairy foods in the foreseeable future.

Turning to fresh meats, the prospects of applying radiation pasteurization are not nearly so dim. At the present time there is no effective and permissible method for processing and preserving fresh meat and yet maintaining its desired color. Radiation pasteurization would offer a means for accomplishing this goal. Even though the meat is to be cooked before consuming, the question may arise as to whether certain harmful microorgan-

isms might be favored by such a processing technique. It is highly doubtful that any such health hazard would arise. To prevent the accumulation of objectionable radiation-induced flavors and odors the radiation dose must be kept to a minimum, especially with beef. To achieve any benefit from such a processing technique the meat would have to be maintained at temperatures below the range that supports the growth of any known human pathogen that might be found in the meat. At temperatures above 10°C. the irradiated meat would spoil quickly as the result of the growth of surviving saprophytes.

Gomberg & Gould (60) recorded the interesting observation that the female *Trichinella spiralis* could be sexually sterilized by subjecting infected pork to $\gamma$-radiation of approximately 20,000 rads. These authors suggested this technique as a possible method of controlling the incidence of trichinosis in humans.

If the prevention of trichinosis were the only purpose of irradiating pork it is doubtful whether such a control measure would be successful. The literature records cases in which some of the more explosive outbreaks of trichinosis in humans results from the slaughter of the family-owned, garbage-fed hog, which is consumed locally in the form of uncooked pork specialty foods. It is unlikely that pork from such animals would receive irradiation.

On the other hand, pork that is intended to be used commercially in foods which receive a light processing insufficient to kill the trichinae must be certified as being free of this health hazard. The certification is granted if the pork is subjected to a prescribed freezing schedule. It is possible that in some instances irradiation may prove to be a more economical precertification procedure than the present conventional methods.

Proctor *et al.* (61) and Nickerson *et al.* (62) demonstrated that the *Salmonella* in eggs could be inactivated by irradiation. A dose of 300,000 rads inactivated *Salmonella* in liquid whole egg pulp or egg white, but one million rads were needed for dried egg white. Hannan *et al.* (6) stated that 500,000 rads inactivated *Salmonella* in frozen egg white. Such procedures would be expected to inactivate *Salmonella* in other foods.

Although some purified proteins and enzymes may be altered by relatively low radiation doses, these substances are generally very resistant as they occur in foods. In accordance with this knowledge, Dack & Wagenaar (63) found the toxin of *C. botulinum* to be very resistant to $\gamma$-radiation. When the initial toxin levels were above 200 MLD, a reduction to below 20 MLD required at least $4 \times 10^6$ rads. Approximately $5 \times 10^6$ rads were required for a similar reduction in potency when MLD levels above 1000 were employed. In cheese with initial potency of 1000 MLD, a reduction of potency to below 20 MLD required at least $7 \times 10^6$ rads.

Dack, Wagenaar & Surgalla (64) found that partially purified preparations of *Staphylococcus* enterotoxin were inactivated by 720,000 rads but

not by 630,000 rads. Monkey feeding tests were employed as an assay procedure for the detection of enterotoxin.

Some concern has been expressed regarding the possibility that radiation-resistant mutants might be encountered, especially among the pathogens, if radiation were generally instituted in food processing. Indeed, Gaden & Henley (65) were able to isolate mutants among two *E. coli* strains by exposing serial subcultures to sublethal doses of ionizing radiation. A third strain showed an increasing susceptibility to radiation injury under the same conditions.

Following essentially the same technique of Gaden & Henley, Wolin *et al.* (66) were unable to detect radiation-resistant mutants from strains of the fresh meat spoilage pseudomonads. Pepper *et al.* (44) and Koh *et al.* (67) were unable to detect radiation-resistant mutants among *C. sporogenes* and *Bacillus pumilus* spores, and *E. coli*, respectively. Whether radiation-resistant mutants will become a problem remains to be seen. It should be recalled, however, that variations in radiation resistance occur among different strains within one species.

## Concluding Comments

The only purpose in subjecting foods to ionizing radiations is to inactivate the potentially harmful and spoilage microorganisms. This can be accomplished in any food. However, in the light of the damaging effects to the food itself with respect to flavor, odor, color, and texture when subjected to sterilizing doses, it is doubtful whether this method of processing will compete with conventional methods of sterilizing foods in the foreseeable future. Even if these damaging effects could be minimized effectively, the food would probably undergo undesirable deteriorative changes if held for prolonged periods of time above refrigerating temperatures. Therefore, such foods as fresh meats would have to be salted, heated, or treated in some other manner in addition to irradiation in order to achieve the ultimate goal. The economic aspects also must be seriously considered if irradiation is expected to gain widespread use in the food industries.

In comparison to other meats, pork is relatively resistant to radiation damage. It seems entirely possible that such foods as bacon or pork sausage (68) can be prepared as stable food items of acceptable quality by irradiation. These foods would not have to receive sterilizing doses to effect a stable item. Other applications for military food rations appear possible.

The more promising application of irradiation in the food industry remains in the field of radiation pasteurization, especially for meats. In this area radiation processing could become a method for preservation where no other effective method now exists. The possibility of certifying pork with regards to freedom from trichinosis hazard warrants further consideration.

Other research has pointed out the usefulness of ionizing radiations in

controlling insect infestations, preventing the sprouting of potatoes and onions, and sterilizing pharmaceuticals, arterial segments for transplants in humans, as well as sutures, bandages, and other medical supplies (59). In view of these possibilities it is entirely reasonable to expect that radiation processing will eventually gain acceptance in the food processing industries.

## LITERATURE CITED


1. Brasch, A., and Huber, W., *Science*, **105**, 112 (1947)
2. Kelner, A., Bellamy, W. D., Stapleton, G. E., and Zelle, M. R., *Bacteriol. Rev.*, **19**, 22 (1955)
3. Kimball, R. F., *Ann. Rev. Microbiol.*, **11**, 199 (1957)
4. Hannan, R. S., *Proc. Soc. Appl. Bacteriol.*, **16**, 88 (1953)
5. Hannan, R. S., *Food Sci. Abstr.*, **26**, 121 (1954)
6. Hannan, R. S., and Thornley, M. J., *Food Manuf.*, **32**, 457, 559 (1957)
7. Bellamy, W. D., Goldblith, S. A., Colovos, G. C., and Niven, C. F., Jr., *Bacteriol. Rev.*, **19**, 266 (1955)
8. Batzer, O. F., and Doty, D. M., *J. Agr. Food Chem.*, **3**, 64 (1955)
9. Goldblith, S. A., and Proctor, B. E., *Nucleonics*, **10**, 28 (1952)
10. Foster, F. L., Dewey, D. R., and Gale, A. J., *Nucleonics*, **11**, 14 (1953)
11. Knowlton, J. A., Mahn, G. R., and Ranftl, J. W., *Nucleonics*, **11**, 64 (1953)
12. Dewey, D. R., Nygard, J. C., and Kelliher, M. G., *Nucleonics*, **12**, 40 (1954)
13. Loeding, J. W., Petkus, E. J., Yasui, G., and Rodger, W. A., *Nucleonics*, **12**, 15 (1954)
14. Duffey, D., *Nucleonics*, **11**, 9 (1953)
15. Goldblith, S. A., Proctor, B. E., Davison, S., Kan, B., Bates, C. J., Oberle, E. M., Karel, M., and Lang, D. A., *Food Research*, **18**, 1 (1953)
16. Bellamy, W. D., and Lawton, E. J., *Nucleonics*, **12**, 54 (1954)
17. Lea, D. E., *Actions of Radiations on Living Cells*, 2nd ed. (Cambridge University Press, Cambridge, Engl., 416 pp., 1955)
18. Hutchinson, F., *Radiation Research*, **7**, 473 (1957)
19. Gunter, S. E., *Bacteriol. Proc.* (*Soc. Am. Bacteriologists*), 49 (1957)
20. Burns, V. W., *Radiation Research*, **4**, 394 (1956)
21. Gunter, S. E., and Kohn, H., *J. Bacteriol.*, **71**, 571 (1956)
22. Hollaender, A., and Stapleton, G. E., *Physiol. Revs.*, **33**, 77 (1953)
23. Doudney, C. O., *Bacteriol Proc.* (*Soc. Am. Bacteriologists*), 49 (1957)
24. Stapleton, G. E., and Woodbury, D. H., *Bacteriol Proc.* (*Soc. Am. Bacteriologists*), 50 (1957)
25. Wolin, E. F., Evans, J. B., and Niven, C. F., Jr., *Food Research*, **22**, 682 (1957)
26. Wood, T. H., and Taylor, A. L., *Radiation Research*, **6**, 611 (1957)
27. Stapleton, G. E., and Eddington, C. W., *Radiation Research*, **5**, 39 (1956)
28. Proctor, B. E., Goldblith, S. A., Oberle, E. M., and Miller, W. C., Jr., *Radiation Research*, **3**, 295 (1955)
29. Proctor, B. E., Goldblith, S. A., Fuld, G. J., and Oberle, E. M., *Radiation Research*, **8**, 51 (1958)
30. Kempe, L. L., *U.S. Atomic Energy Commission Report TID 7512*, 253 (1956)
31. Williams, N. J., and Kempe, L. L., *Appl. Microbiol.*, **5**, 366 (1957)

32. Stapleton, G. E., Billen, D., and Hollaender, A., *J. Cellular Comp. Physiol.*, **41**, 345 (1953)
33. Proctor, B. E., Goldblith, S. A., Bates, C. J., and Hammerle, O. A., *Food Technol.*, **6**, 1 (1952)
34. Schultz, H. W., Cain, R. F., Norden, H. C., and Morgan, B. H., *Food Technol.*, **10**, 233 (1956)
35. Niven, C. F., Jr., and Chesbro, W. R., *Antibiotics Ann.*, (1957)
36. Koh, W. Y., Morehouse, C. T., and Chandler, V. L., *Appl. Microbiol.*, **4**, 143 (1956)
37. Morgan, B. H., and Reed, J. M., *Food Research*, **19**, 357 (1954)
38. Niven, C. F., Jr., *U.S. Atomic Energy Commission Report TID 7512*, 265 (1956)
39. Drake, S. D., Evans, J. B., and Niven, C. F., Jr., *Food Research* (In press)
40. Drake, S. D., Evans, J. B., and Niven, C. F., Jr., *Am. Meat Inst. Foundation* (Unpublished data)
41. Berk, R. S., *Am. Meat Inst. Foundation* (Unpublished data)
42. Anderson, A. W., Nordan, H. C., Cain, R. F., Parrish, G. Duggan, D., *Food Technol.*, **10**, 575 (1956)
43. Bridges, A. E., Olivo, J. P., and Chandler, V. L., *Appl. Microbiol.*, **4**, 147 (1956)
44. Pepper, R. E., Buffa, N. T., and Chandler, V. L., *Appl. Microbiol.*, **4**, 149 (1956)
45. Schmidt, C. F., *Continental Can Co. Research and Develop. Dept. Bull.* (Unpublished report)
46. Kempe, L. L., *Appl. Microbiol.*, **3**, 346 (1955)
47. Kempe, L. L., Graikoski, J. T., and Bonventre, P. F., *Appl. Microbiol.*, **5**, 292 (1957)
48. Kan, B., Goldblith, S. A., and Proctor, B. E., *Food Research*, **22**, 509 (1957)
49. Clark, J. H., *Am. J. Roentgenol. Radium Therapy*, **40**, 501 (1938)
50. Kirn, J. F., Urbain, W. M., and Czarnecki, H. J., *Food Technol.*, **10**, 601 (1956)
51. Schweigert, B. S., Niven, C. F., Jr., Doty, D. M., and Kraybill, H. R., *Am. Meat Inst. Foundation Circ. No. 10 (1954)*
52. McLean, R. A., and Sulzbacher, W. L., *J. Bacteriol.*, **65**, 428 (1953)
53. Thornley, M. J., *J. Appl. Bacteriol.*, **20**, 286 (1957)
54. Nickerson, J. T. R., Lockhart, E. E., Proctor, B. E., and Licciardello, J. J., *Food Technol.*, **8**, 32 (1954)
55. Gaden, E. J., Jr., Henley, E. J., and Collins, V. P., *Food Technol.*, **5**, 506 (1951)
56. Nickerson, J. T. R., Proctor, B. E., and Goldblith, S. A., *Am. J. Public Health*, **43**, 554 (1953)
57. Niven, C. F., Jr., and Chesbro, W. R., *Proc. Conf. on Research, Am. Meat Inst., 8th Conf.*, 47 (1956)
58. Jordon, R. T., and Kempe, L. L., *Proc. Soc. Exptl. Biol. Med.*, **91**, 212 (1956)
59. Proctor, B. E., and Goldblith, S. A., *Am. J. Public Health*, **47**, 439 (1957)
60. Gomberg, H. J., and Gould, S. E., *Proc. Intern. Conf. Peaceful Uses Atomic Energy, 1st Conf.*, **15**, 251 (1955)
61. Proctor, B. E., Joslin, R. P., Nickerson, J. T. R., and Lockhart, E. E., *Food Technol.*, **7**, 291 (1953)
62. Nickerson, J. T. R., Charm, S. E., Brogle, R. C., Lockhart, E. E., Proctor, B. E., and Lineweaver, H., *Food Technol.*, **11**, 159 (1957)

63. Dack, G. M., and Wagenaar, R. O., *Proc. Inst. Food Technologists, 15th Conf.*, 28 (Columbus, Ohio, 1955)
64. Dack, G. M., Wagenaar, R. O., and Surgalla, M. J. (Unpublished data)
65. Gaden, E. L., Jr., and Henley, E. J., *J. Bacteriol.*, **65**, 727 (1953)
66. Wolin, E. F., Drake, S. D., Evans, J. B., and Niven, C. F., Jr. (Unpublished data)
67. Koh, W. Y., Morehouse, C. T., and Chandler, V. L., *Appl. Microbiol.*, **4**, 153 (1956)
68. Proctor, B. E., Nickerson, J. T. R., Licciardello, J. J., Goldblith, S. A., and Lockhart, E. E., *Food Technol.*, **9**, 523 (1955)

# CHEMICAL DISINFECTANTS[1]

By Charles R. Phillips and Benjamin Warshowsky
*U. S. Army Chemical Corps,*
*Fort Detrick, Frederick, Maryland*

It has been ten years since the subject of chemical disinfectants was last covered in the *Annual Reviews of Microbiology* (1). No attempt is made in this review to include all the data which have appeared since that time as the volume of work published in the last decade and the amount of space allotted for this review makes this an impossibility. Rather, this article is intended to serve more or less as a status report on chemical disinfection as of the present moment, with passing comment on certain areas in which the greatest recent activity has taken place. As such, it represents to a large degree the bias of the authors, who arbitrarily selected that material which was of most interest to them.

## INTRODUCTION

The term "chemical disinfectant" cannot even be defined without a certain amount of bias. The field covered under this category is not the same to all people. For example, most investigators would agree that the subject should include all those chemicals which are applied to inanimate objects or materials to lessen their microbiologic contamination. Too literal an interpretation of such a definition, however, would necessitate a discussion of such a simple compound as water. Lysis of microorganisms occurs in distilled water and the different effects observed in an autoclave and a hot-air oven of the same temperature would certainly indicate that water could be considered a chemical disinfectant in these cases.

The authors have followed the general practice in this field of including the main compounds used to treat inanimate materials, and some of those compounds, such as mercurials, used externally in medicine. Excluded are those substances used as internal medicants, as well as food preservatives, and the whole range of compounds, usually antifugal, used in agronomy or horticulture to treat plant diseases.

When one considers the present-day status of chemical disinfectants, the most salient general observation that can be made is that the science is still largely an empirical one. Much is known and volumes have been published on such subjects as which compounds destroy which microorganisms under specified sets of conditions. Little is known (and what has been published is mostly speculation) on the exact phenomena occurring in these processes. Some comment on what has been stated concerning mechanisms

[1] The survey of the literature pertaining to this review was completed in December, 1957.

of action will be given in the following sections, in which various types of disinfectants are discussed. One general statement should be made now concerning the apparent reasons for the poor state of knowledge in this field. The chemistry of the various compounds used as chemical disinfectants is for the most part well known, together with the type of reactions which they undergo with simple organic compounds. What is not well known is the complex chemistry of the microorganisms themselves, the organization of these chemicals in the organism's cellular structure, and the physiologic processes going on within the living organism. Such information is slowly being evolved, not by those working in the field of disinfection but by those interested in biochemistry, enzymology, immunology, cytology, physiology, and other related fields. Perhaps not until these scientists have developed their areas more perfectly can anyone expect that definitive information can be established concerning the mechanisms involved in chemical disinfection.

## CRITERIA OF DEATH

It is interesting that the microbiologist has no clear-cut definition of life and death itself. The most common criterion used to determine whether any single organism is living or not is to place it in a favorable environment, and then come back some hours later and observe whether or not it has proliferated and produced descendants. Few scientists would care to apply such a criterion of life to higher organisms, such as, say, man. Indeed, grave doubts have arisen as to how accurate it is to apply it to such a simple organism as a bacterium. For example, it is hardly necessary to refer to the volume of older work which established that many processes thought to be bactericidal were merely bacteriostatic. This was first dramatically shown with the mercurials (2) whose action was so easily reversed by sulfhydryl compounds, as will be discussed later. Nowadays hardly any investigator publishes material on a chemical disinfectant without stating what he has done to neutralize it afterward to assure that he treated organisms are killed, not merely inhibited.

A more puzzling and more recent activity in retrieving microorganisms from apparent death is the work done in the past decade on photoreactivation. This phenomenon was first described by Kelner with *Streptomyces griseus* (3), *Escherichia coli*, and other organisms (4). He demonstrated that visible light of wavelengths under 5100 A would cause the recovery of microbial cells from ultraviolet-induced damage which would otherwise be fatal. Many other papers have followed confirming the original observations; these will not be reviewed here other than to quote a recent article by Giese, Iverson & Sanders (5) which stated, "Unfortunately the basic reactions in photoreactivation remain elusive. What they are not is easier to determine than what they are." Death is shown again to be a phenomenon difficult to measure when dealing with microorganisms.

While the killing of microorganisms with ultraviolet light or other radia-

tions is not the subject of this review, and the matter was mentioned only to point out uncertainties in determining death, there was an extension of this work into the field of chemical disinfectants which is worthy of discussion. Heinmetz and co-workers who first, following Kelner (3, 4), reported on light reactivation of ultraviolet-"killed" organisms (6), subsequently published that the same reactivation could be obtained by incubation in sodium pyruvate (7) and other Krebs cycle metabolites (8). They then reported that the same reactivation with metabolites could be obtained with heat and chlorine-"killed" organisms (9). Thus, these workers offered yet another mechanism by which microorganisms, presumably killed either by ultraviolet radiation, heat, or chemical action, might be shown to be still viable.

This latter method, that of reactivating so-called killed organisms by a treatment with metabolic components, was not allowed to go unchallenged. The attack centered about the demonstrable fact that the organisms being studied would multiply in the "reactivating" solutions. The question arose whether any cells had been "reactivated" or whether a few scarcely unnoticed survivors had multiplied to quite noticeable proportions during the treatment. Garvie, who first studied this phenomenon (10, 11, stated, "It is important to know whether bacteria can be reactivated after treatment by disinfectants; no evidence so far available proves that in fact they can be." Chambers, Tabak & Kabler (12) came to the same conclusion. When small numbers of survivors remained following heat or chlorine deactivation, much larger numbers were recovered following metabolite reactivation. When the original suspensions were truly sterile following heat or chlorine treatment, they were also sterile following incubation in metabolite suspensions. These authors conclude that "Data obtained in this investigation do not indicate that these 'growth factors' can restore the metabolic cycle in inactivated cells and subsequently restore cellular viability in terms of ability to multiply." The criterion of death in all these studies is again the demonstration of whether or not multiplication will occur when the organisms are placed in a favorable medium.

This difficulty in ascertaining when death has occurred in chemical disinfection has had other ramifications. As long ago as 1908 when Chick (13) first started publishing on the subject, it had been realized that when large numbers of microorganisms are subjected to chemical disinfection, heat, radiation or other lethal factors, the count drops off approximately exponentially with time. This is equivalent to saying that in any given time period a definite fraction of the population will die, and in the next similar time period the same fraction of the survivors will die, and so forth. The process is thus similar mathematically to a monomolecular chemical reaction, or to the radioactive decay of an unstable chemical isotope. Bacteriologists call this the logarithmic death rate, since if the logarithm of the number surviving in any experiment is plotted against the exposure time, a straight line can usually be drawn through the points. Since this treatment

usually fits observed facts within experimental error and is such a convenient way of treating disinfection data, scant attention is usually paid to cases of poor fit, or to the fact that no good rationale has been developed as to why such a mathematical formula should logically be followed in the disinfection process. Many cases of poor exponential fit have been published, extending back indeed to Chick herself, who was the first to emphasize the usual generality of the observation. In many of these cases, small but significant numbers of surviving organisms are found after extended treatment times, where an extrapolation of the exponential death function would have indicated a very low probability of any being found in the volume tested.

One extremely well publicized episode, and one highly critical paper which summarized the whole proceedings, can best be utilized to illustrate the difficulties which can ensue from this uncertainty as to when death of microorganisms has occurred, and the rate or order in which this process takes place. We refer to the experience in this country with the Salk poliomyelitis vaccine, and the infections which occurred in its large-scale distribution and use in 1955, and to the summary of this experience published by Meier in May of 1957 (14). In the preparation of a vaccine from a live virulent culture it is, of course, important that all the virulent organisms be killed, or at least if any are left alive that they be too few in number to cause an infection. When this killing is done chemically, with formaldehyde in this case as with many other vaccines, the procedure is essentially one of chemical disinfection. It is also important that the chemical treatment not be unnecessarily prolonged or drastic, or else the antigenic value of the killed vaccine will be lessened. No vaccine was more eagerly awaited, nor was any vaccine more precipitously moved from laboratory to full-scale commercial production and universal application than was the Salk vaccine. When a number of infections occurred which became more and more attributable to certain commercial lots of the vaccine, the issue became not just a scientific one but one which was fought out daily in the press and other media of public news dissemination. As Meier (14) sums up the situation, the large-scale 1954 field trial (15) (using a few commercially prepared lots) had indicated the safety of the vaccine, and Salk's own report (16), placing reliance on the validity of the exponential death process, had indicated that on theoretical grounds the vaccine should be absolutely safe. Meier comments

> Unfortunately, Salk's 1954 paper did not give detailed evidence in support of this theory, and the state of knowledge at this time, as judged from other sources, makes the validity of these assumptions appear questionable. . . . Even if the rate of inactivation appeared constant over the observable range, the extrapolation beyond the observations would be questionable. Actually some of the data presented by Salk in February 1955 (17) show a non-constant rate of inactivation.

Even if the cherished trust which microbiologists have placed in the

logarithmic order of death for the past 50 years did not provide absolute safety with the Salk vaccine, it might have been expected that the sterility testing instituted on a random sampling basis on the commercial lots of vaccine might still have provided protection to the population to whom the vaccine was being administered. This sterility testing was done with tissue cultures from monkey kidney. Meier quotes data (18) showing that the chance of one virus particle infecting such tissue was only 1 in 200, with some question of the validity of this estimate (19) at the time that the Salk vaccine was being tested in 1954, and that refined measurements reported after the infections occurred raised this figure only to about 1 in 50 (20). Moreover he states, "There was, furthermore, no evidence on the relative sensitivity of children and tissue-culture preparations." In connection with more recent tests he states "Evidently something happens in the process of treatment with formaldehyde which reduces the ability of a virus to infect a tissue-culture preparation more than its ability to infect cortisone-treated monkeys." The latter, incidentally, are incorporated in present safety tests.

To the present authors, this widely publicized controversy can only emphasize that in chemical disinfection, death and the rate at which it occurs with microorganisms is still inadequately defined, even after a half century or more of investigation on the subject.

## TEST METHODS

It is hardly surprising, in view of the uncertainty as to the exact mechanisms involved in chemical disinfection, and uncertainties as to whether or not death has occurred as a result of their action, that little common agreement exists on what methods should be used to test and evaluate disinfectants. This is not because too little work has been done on the subject, but rather because of the enormous volume of work which has been done, the multiplicity of test methods which have been proposed, and the difficulty of determing the meaning of the information received from these test results. A recent text, edited by Reddish (21), contains eight chapters under the general section of "Methods of Testing," three by himself and the others by different authors, which total almost 200 pages and include several hundred references. No attempt will be made in this article to cover testing in specific or full detail.

Attempts to standardize test methods extend back at least to 1903 when Rideal & Walker (22) first proposed the phenol coefficient test. With many modifications this is the most widely used standard test today. In this country it is recommended by the U.S. Food and Drug Administration (23) and it is an official test of the Association of Official Agricultural Chemists (24).

The phenol coefficient and other more or less standard test methods are covered in great detail in Reddish's book (21), which also includes the methods which have appeared since the last review in this publication by

Wyss (1). Reddish (21) sums up the pheno coefficient discussion as follows:

> In a sense it (the modified phenol coefficient test) is only a "screening test," intended to separate preparations which are not actively germicidal from those which are positive and quick-acting germicides. This is important, however, and is sufficient justification of the test.

Elsewhere he states, "Those preparations which do pass the test within 5 minutes or less must then be submitted to further study under practical and clinical conditions."

It is this last sentence which is particularly important. More and more we are forced to realize that there is no hope of getting the performance data we desire on any chemical disinfectant from a single or relatively few standard comparative tests. Perhaps the best we can obtain with certainty are data which clearly show how one particular chemical disinfectant performs under certain prescribed conditions similar to those for which it was intended to be used. Certainly no one test could ever be devised which will compare the relative efficiency of a mercurial in healing a child's skinned knee with that of chlorine in rendering river water potable, or of ethylene oxide gas in sterilizing nonautoclaveable plastic Petri dishes.

## MERCURIALS

One of the most active controversies on the subject of validity of test methods, which took place in the past decade, centered about mercurials. The debate concerned evaluating the efficiency of such compounds in applications similar to the above-mentioned problem of the child's skinned knee. The paper of Morton, North & Engley (25), which appeared in 1948, in effect set off the controversy. Because of the implications of this beyond the specific disinfectants which were involved, mercurials as a group will be discussed first in this article, and in a little more detail than may be given to other sections.

The value of these compounds has varied from general acceptance to conditioned malignment; the pendulum finally appears to be settling in an area where recognition can be given to their capabilities and limitations. Despite specific experimentation purporting to show that compounds of mercury are, in general, not bactericidal, the fact exists that they have been used successfully for many years to help prevent sepsis.

The basis for the loss of confidence in the mercurials as disinfectants stems from the observations that sulfides (2, 26), mercapto compounds (27), and serum (28, 29) reversed the apparent bactericidal properties of mercury compounds when added to cultures of microorganisms which had been "killed" by these agents. *In vitro* studies to demonstrate the inability of mercurials to act as germicides involve the use of media containing thioglycollate (30) or other mercury-binding groups (31). Under these circumstances the sulfhydryl group (and to a lesser extent other available functional groups) reacts stoichiometrically with combined, or free, mercury

cations to form a less dissociated complex, thereby neutralizing the antiseptic efficiency of the mercury.

While the specific mechanism of action of mercurials has not yet been precisely defined, considerable evidence of a general nature is available to provide a working knowledge of the phenomena involved (32, 33, 34). The fundamental reaction between microorganisms and ionizable mercury compounds, or organic mercurial cations, is the combination with essential enzymatic constituents of the bacterial cell presumably by interaction with available sulfhydryl groups (35). This interaction, it is postulated, results in an inhibition of enzymatic activity which interferes with vital life processes of the cell. No conclusive information exists to establish whether the disinfectant activity of mercury is due to reaction at a particularly sensitive site of a critical enzyme or whether more than one enzyme is affected. Available data seem to favor the hypothesis that it is a summation of effects on numerous enzymes which depends upon sulfhydryl groups for their activity (36).

The fact remains, however, that mercury does inhibit growth and reproduction of microorganisms and that, depending on such factors as length of time of exposure, concentration of the disinfectant, and the temperature, it is either bactericidal or bacteriostatic. As discussed by Lamanna & Mallette (37), the disinfecting action of mercuric ions probably occurs in three phases. The first is probably a diffusion stage during which the mercury ion penetrates into the bacterial cell; this stage is reversible by simple dilutions such as repeated washing with water or an isotonic solution. The second stage is a reaction with sulfhydryl groups which is reversible in the presence of substances capable of competitively displacing the mercuric ion. There then occurs a third, final stage which is irreversible.

Morton, North & Engley (25) attempted to show that in order for a mercurial to be antiseptic, it must be germicidal. Their basis for questioning the effectiveness of the organic mercurials was the fact that mice usually died following an intraperitoneal injection of a culture of hemolytic streptococcus which had been exposed for 10 to 15 min. to aqueous concentrations of several widely used commercial organomercurial compounds; and the organisms were isolated from heart's blood after death of the mice. Furthermore, evidence was presented to show that the organisms, rendered static by the mercury treatment, were still infectious even without neutralization prior to injection. The authors maintained that the commercial preparations were not reliable germicides for the inactivation of infectious organisms.

The evidence presented by Morton, North & Engley (25) was so substantial that it prompted the Council on Pharmacy and Chemistry of the American Medical Association to challenge the manufacturers to submit experimental verification of the usefulness of their products.

In direct response to this request a number of papers have been published clarifying somewhat the limitations of the mercurials and defining their roles as antiseptics. Brewer (38) presented data on the use of Mer-

curochrome to show that the infectivity of several different bacteria is definitely impaired by the action of the mercurial. However, some question exists regarding the virulence of the strain of hemolytic streptococcus that was used. Powell (39), using sodium ethylmercurithiosalicylate (Merthiolate), pointed out that the environmental factors such as temperature, time of contact, and quantity of bacteria involved influence the effectiveness of mercurials. Other authors (40, 41) reported data that, while not directly contradictory to those of Morton, North, and Engley, demonstrated the value of organomercurial preparations in reducing infections under selected conditions of use. Mortensen *et al.* (42) found 1:1000 buffered solution of Merthiolate useful for maintaining sterility of arterial grafts obtained under aseptic conditions. Davisson *et al.* (43) established that Merthiolate successfully preserved poliomyelitis virus vaccine without loss of antigenicity if the sodium salt of ethylenediamine is added.

From the foregoing it is apparent that the antibacterial activity of mercurials is in reality a relative measurement and is dependent on the test method used. Engley (29) proposed a paper-disc assay method similar to that used with antibiotics to compare the bacteriostatic activities of mercurials. By this technique, zones of inhibition were obtained with various mercury compounds using nutrient agar, but no inhibition was noted in the presence of media containing thioglycollate or serum. A direct *in vivo* method for evaluating the antiseptic properties of germicides was employed by Brewer & McLaughlin (41) using tetanus spores as the test organism. The procedure consists of injecting into mice 10 to 100 minimum lethal doses of the spores followed by injection of the test antiseptic at the same site. The degree of protection is a measure of the effectiveness of the disinfectant. Numerous other *in vitro* and *in vivo* tests have been published for evaluating the antiseptic efficiency of proposed chemical disinfectants (21). Space does not permit a detailed review at this time; however, the consensus of responsible investigators in discussing the interpretation of results obtained with the mercurials by such methods appears to be that no single test has yet been devised which reflects the true effectiveness of these chemical compounds. Brewer (44) summarized the status of the mercurials as follows:

> The scientific literature of the last 50 years abounds in efforts to show the limitations of the mercurials. The most severe criticism is that the mercurials can be neutralized by SH compounds found in the body. This criticism can be borne out by laboratory data; however, to suggest that mercurials cannot act as antiseptics simply because they are easily neutralized by substances found in the body, such as glutathione, is like suggesting that the sulfonamides are not satisfactory chemotherapeutic agents, since they are easily neutralized by p-aminobenzoic acid or that penicillin will not work orally since *E. coli* and other intestinal organisms produce penicillinase which inactivates this antibiotic. It is readily agreed that the inherent toxicity of the mercurials removes them from the realm of usefulness as systemic chemotherapeutic agents and their inability to kill spores prevents their use as

germicides for sterilizing instruments. Fortunately the mercurials will act as bacteriostatic agents to both spore-forming and non-sporulating organisms and thereby prevent infection. . . . This work does not suggest that any of the mercurial antiseptics can be depended on to prevent infection by spores in deep puncture wounds, gangrenous areas or other conditions where antitoxin should be relied upon. One should rely on clinical experience in such matters and clinical records bear out the view supported by Lister's original record based on Koch's work that contaminated wounds treated promptly with antiseptics do not suppurate.

## SILVER

Considerable literature has been produced concerning the use, dynamics, and physicochemical forms of silver as a disinfectant. However, an examination of the data available indicates that the specific mechanism of the oligodynamic action of silver still remains undefined.

What has been shown experimentally is this: that colloidal silver in some as yet unresolved active state is an effective disinfectant for water and surfaces in extremely small concentrations (microgram quantities) when applied either as a powder, solution, or ointment, and is nontoxic to man even in rather high concentrations. Numerous theories have been advanced to explain the observed antimicrobial activity of silver but no single proposal has emerged with any preponderance of experimental support. For example, an early theory suggests that the oligodynamic effects of silver are due to simple chemical solution and diffusion (45, 46). Later the catalytic effect of the metal, with a subsequent toxicity to the cell, was proposed (47, 48) as a mechanism. Another explanation is that adsorption of colloidal metals or metallic ions on the cell surface is the basis for the disinfection (49 to 52). Neisser & Eichbaum (53) and Goetz (54) concluded that the oligodynamic metals act through the formation of metal protein complexes with the living cell or its enzyme systems.

Several investigators (55, 56, 57) observed that combinations of metals often increased the activity of pure silver and that an electropotential effect was involved. Some evidence also has been presented which indicates that the causative effect of silver activity is due to the formation of (*a*) organometallic salts (53, 58); (*b*) metallic oxides (58, 59); (*c*) metallic chlorides (60, 61, 62); (*d*) free metallic ions (54, 63); or (*e*) complex silver ions (64). Whereas silver has been demonstrated to inhibit the action of specific enzymes (65, 66, 67), this alone is not always responsible for death of the organism (68). The present authors tend to summarize the observed oligodynamic effects by pointing out that finely divided metallic silver presents an excellent oxidative catalytic surface.

A survey of the variety of oligodynamic silver preparations reveals an assortment of processes and products which have had wide application. These include the Cumma System (69) which is an electrolytic method for the production of chlorine and silver ions; the Matzka Process which produces germicidal concentrations of silver ions in a medium by an electro-

potential difference between a silver-gold alloy anode and a nickel or aluminum cathode; Katadyn silver (56) which is a preparation of a spongy metallic silver and a metal below silver in the electronegativity series; various silver-coated adsorbents such as sand (70), filter elements (71), organic fibers (72), plastic granules (73), resins (74, 75), or carbon (76); Fissan-silver, a preparation containing silver linked to an unstable milk albumin mixed with a Fissan colloid (77); Movidyn, an aqueous suspension of a colloidal silver preparation (52, 78); and O-Silver, a solution of oligodynamic silver (79). Other preparations of interesting activity and application were developed by Mendenhall (80), Goetz (81), Lindenmeyer (82), and Edlinger (83).

The efficacy of these preparations has been succinctly reviewed by Romans (79) and need not be elaborated at this time. In general, it can be shown that the rate of kill of microorganisms by these preparations varies with the concentration of silver, the temperature of the medium, the presence of inorganic and colloidal substances, and the number and type of organisms involved. In regard to the latter characteristic, Süpfle & Werner (84) observed that Katadyn silver did not destroy the viability of an aqueous suspension of *Bacillus mesentericus* spores after five weeks of contact. However, it was suggested that the noted limitations should not preclude the consideration of silver treatment of drinking water because the only infection by pathogenic spores which might be transmitted by water was anthrax. Kliewe, *et al.* (64) found that *Salmonella typhosa* on agar surfaces exposed to silver chloride aerosols was killed within 1 to 2 min., *E. coli* within 2 to 3 min., staphylococci within 10 min., and anthrax spores within 15 to 30 min. Bactericidal action was not decreased by the presence of serum, blood, saliva or meat. Movidyn was evaluated by Hoffman, *et al.* (78). They demonstrated that the silver was more active than silver nitrate and that it was bactericidal; 0.1 p.p.m. killed *Serratia marcescens* in 1 hr., *S. typhosa* in 3 hr., *Micrococcus pyogenes* var *aureus* in 5 hr., and *E. coli* in 7.5 hr. Chambers, Chambers & Kabler (85) determined the effects of some environmental variants on the bactericidal action of Movidyn. Optimum efficiency was observed at pH 8.5 to 9.0 and the efficiency decreased with decreasing temperature. Dunklin (52) presented data to show that Movidyn is more active than Katadyn silver and stated that, from rate-of-kill curves, the action appeared irreversible. Paper fibers treated with O-Silver prevented and cured infantile diaper rash; O-Silver ointment was effective for burns and infected wounds; and the aqueous form was recommended for preoperative skin preparation.

## QUATERNARY AMMONIUM COMPOUNDS

The antimicrobial action of cationic surface-active detergents such as the quaternary ammonium compounds (Quats) has been ascribed either to their effect upon enzyme inhibition, protein denaturation, or to the influence

of these compounds on the permeability of cell surface. Hotchkiss (86), Gale & Taylor (87, 88), and Salton (89, 90) presented data to support the thesis that a close correlation exists between the germicidal activity of Quats and cell lysis as measured by the release of specific cellular constituents into the surrounding medium. Newton (91) used absorption at 260 mμ to measure the liberation of nonspecific cellular substances. Knox *et al.* (92) concluded that enzyme inactivation was the mechanism leading to the death of the organisms. Stedman, Kravitz & King (93), using $C^{14}$-labelled *S. marcescens,* attempted to resolve these different opinions. These authors observed that at high concentrations of quaternary ammonium compounds and high cell density, the germicidal effect caused by lysis may be significant. At low concentrations of disinfectant the "cessation of energy-yielding reactions, interference with growth and reproduction, and other effects not measured may be playing major roles, and mechanical leakage of nonspecific cellular material is of secondary importance."

Although the antimicrobial activity of the Quats has been demonstrated principally against vegetative nonsporulating organisms, some activity has been observed on other groups. No significant activity of aqueous solutions of these disinfectants was noted against virulent strains of *Mycobacterium tuberculosis* (94, 95); however, tinctures of some Quats gave reasonably high tuberculocidal activity as measured by infection-prevention tests. Although viruses, in general, are more resistant than bacteria or fungi to the action of Quats (96, 97), Shaughnessy & Zichis (98) noted the efficacy of appropriate applications of aqueous Quat solutions to wounds containing rabies virus.

Because of their relatively low toxicity (99, 100), the Quats have found extensive application as sanitizers in eating and drinking establishments (101, 102), dairy industries (103, 104), and various food processing plants. Limited use of Quats also has been made for disinfecting skin and as preservatives for drug formulations (105). In the latter instance, caution must be exercised to ascertain that no undesirable interaction occurs between the preservative and any of the constituents of the preparation.

## PHENOLICS

Phenol itself is probably the oldest chemical disinfectant, and certainly the one about which most has been written. For the last half century or more it has been the one to which most disinfectants have been compared, as was discussed earlier. In spite of its historical significance, it is not widely used as a disinfectant today. This is not true, however, of its chemical derivatives.

On this subject the synthetic organic chemists have had a holiday. The phenol chemical structure offers five spots upon which substitutions can be made, and upon these locations in the benzene ring, aliphatic, aromatic, halogen, or other substitutions can be, and have been, made in infinite

variety, and subsequently tested for bacterial activity. Substituted simple phenols, di- and tri-hydroxy phenols, *bis*-phenols, and their variously substituted analogues have been synthesized and tested. Within the last year Klarmann & Wright (106) have reviewed the whole field of phenolic compounds in full detail, and Cade & Gump (107) have done similarly for the *bis*-phenols.

Of particular interest is the fact that most substitutions on the phenolic structure increase its bactericidal activity, often many hundredfold as measured by the phenol coefficient test. The substitutions also affect many of the chemical properties of phenol such as its electrolytic dissociation and its water solubility, hence affecting its distribution ratio between aqueous and nonaqueous phases in favor of the latter. Thus the type of substitutions could affect the amount of the chemical that would be picked up by the lipoid constituents of the bacterial cell (108, 109), and also could affect the activity of the phenolic group itself, which presumably accounts for the bactericidal activity of this large series of related compounds. Perhaps the relatively low solubility of these compounds is to a certain extent responsible for their surprisingly high concentration exponents (110, 111, 112), (i.e., their disproportionately increased activity as concentration is increased) which might account for more of the compounds being absorbed by the bacterial cells as their concentrations approach saturation. No such simple relationship as solubility could account, however, for the diverse effects observed in various substitutions, nor could it begin to explain the species selectivity of many of the phenol derivatives. The latter effect is best illustrated by some older investigations of Klarmann & Shternov (113) who showed that as various aliphatic groups from methyl through heptyl were substituted into the benzene ring, the bactericidal activity increased regularly with increased molecular weight with respect to *Staphylococcus aureus, M. tuberculosis,* and *Candida albicans,* but with *S. typhosa* the same effect was noted only up to the amyl substitution. The hexyl and heptyl substituted phenols were less effective than the amyl against this organism, and showed surprisingly lower phenol coefficients towards it than they did to the other three organisms. This is not an isolated observation. Many of the substituted phenols differ markedly in their relative effectiveness against different organisms.

Two different phenomena have been studied which bear upon the mechanism of action of phenol and its derivatives. The compounds apparently affect the cell wall (114), causing lysis (115) and the loss of amino acids (88) and other essential metabolites (116) from the cell. Other studies have shown that these compounds cause definite inhibitions of specific enzyme systems, such as the dehydrogenases (117, 118) and oxidases (119). Either of these activities could be lethal.

One of the more recent and active developments in the field of the phenolic compounds has been their incorporation in germicidal toilet soaps.

The *bis*-phenol, G-11, (Hexachlorophene), in particular has been widely used for this purpose. This development has recently been reviewed in detail by Cade and Gump (107).

## HALOGENS

The halogens, both chlorine (120) and iodine (121), have been recently reviewed. The subject was also extensively covered by Wyss (1) in the last review on disinfectants which appeared in the *Annual Review of Microbiology*. The present authors, at the risk of slighting recent investigators in this field, are inclined toward no further coverage of these compounds. The halogens, of course, in all their various forms, act primarily as oxidants and moreover can directly halogenate many positions in complex organic structures. The effect of concentration, temperature, pH, oxidation-reduction potential, and the presence of extraneous organic material have been extensively covered in the literature. The newer forms in which these elements are available and are used effectively are covered in the recent reviews. Iodine especially is now available in many new forms, particularly in combination with solubilizing agents or carriers as with the iodophors, or in the form of new chemical compounds, rather than as dissolved free iodine. The increase in the number of forms in which it is available has resulted in widened applications. Readers are referred to Gershenfeld (120) for a recent excellent summary of this active field.

## GASEOUS STERILIZATION

The subject of gaseous sterilization is really an old one. It had been so inactive until the past decade, however, that it was not mentioned at all in the Wyss (1) review. Some 20 pages were devoted to the subject in a review by Phillips (122) which appeared nine years later in Reddish's text (21). Gaseous sterilization is really merely a specialized field of chemical disinfection, the disinfectants being chemicals in the gaseous state rather than liquids and solids in liquid solution. The intriguing advantages of such a system are: simplicity in operation, in that a gas distributes itself freely throughout an inclosed space while a liquid does not; ability to penetrate into areas not readily attacked by liquids; avoidance of damage to heat- and moisture-sensitive materials; and easy removal of excess disinfectant by simple airing. Such potential advantages aroused interest in the subject half a century or so ago. It was the failure to find a compound which met the majority of these ideal characteristics that led to the inactivity in the field up to the last decade.

The older work extends back into the late 1800's when the public health practice of fumigating sick rooms was widely practiced. Sulfur dioxide and chlorine had first been tried for such purposes, but they were highly damaging to the materials within the rooms, even when used as dilute gases. It was not until 1894 that formaldehyde, the bactericidal effect of which in

aqueous solution had been discovered some few years previously, was first proposed for use in the vapor phase, so nearly simultaneously, by several authors that it is hard to decide who should be given first credit for the discovery. Hundreds of articles on the use of formaldehyde vapor for disinfecting enclosed rooms appeared in the scientific literature within the next two decades. These papers were concerned not only with the bactericidal effect of formaldehyde vapor, but with such practical considerations as means of releasing formaldehyde into the rooms, and also with surveys of how effective such procedures were in practical application. This literature on formaldehyde gradually dropped off when the practice of terminal fumigation of sick rooms was slowly abandoned, beginning about 1910. One reason for discontinuing these practices lay in the fact that, as practiced, such fumigation often failed to sterilize. Another, perhaps more important reason, was that medical opinion was finding such treatments unnecessary, and discovering other, more effective ways of combating infectious disease. All of this earlier work on formaldehyde was summed up in 1939 in an excellent monograph by Nordgren (123), together with his own experimental data on the bactericidal action of gaseous formaldehyde.

While formaldehyde is seldom used today for the terminal fumigation of sick rooms, various industrial applications have continued. It can be used in vacuum chambers (124) to treat various types of material, and also as a gas to sterilize large enclosed areas such as laboratories or warehouses. Present-day practice in the latter instance (122) is as follows: One milliliter of liquid formalin (37 per cent HCHO) is introduced for each cubic foot of space to be treated, either by heating the formalin to boiling or by passing it through such a fine nozzle that all droplets vaporize before they can settle out. The temperature of the space to be treated must be kept above 20°C. and, if the relative humidity is below 70 per cent, it must be raised by the introduction of additional water vapor. After six hours or more the area is opened and ventilated to remove formaldehyde vapor.

This procedure is only moderately successful. While readily exposed surfaces are indeed sterilized by this treatment, penetration of the gas into relatively protected areas or into porous materials is poor, and contamination often persists in these spots. Moreover, prolonged airing (several days or more) is required to remove highly irritating residual formaldehyde from the treated enclosure.

The reason for both of these disadvantages lies in the fact that formaldehyde, although introduced as a gas, does not remain long in the gaseous state as indicated by chemical sampling of the atmosphere. The concentration of formaldehyde in the air drops quickly to far below equilibrium concentrations. The gas is adsorbed on readily available surfaces, so that in effect we are using a gaseous mechanism to produce a thin film of polymerized formalydehyde or formaldehyde dissolved in moisture on these surfaces. This mechanism accounts for the poor penetration into porous

materials or inaccessible areas, and also accounts for the very long time required for this adsorbed film to revaporize and be removed by airing. Thus the procedure is in reality only a quasi-gaseous one.

Another application that might be called quasi-gaseous sterilization, lies in the use of chemical aerosols intended to disinfect the air and hence lessen respiratory infections in enclosed areas such as school rooms, theaters, barracks, and other places where large numbers of people congregate. The principle involved is the vaporization or atomization of small quantities of bactericidal compounds which have such low vapor pressure that the areosol in essence saturates the air. The amounts involved are too low to be objectionable to people occupying the area but are sufficient to kill air-borne microorganisms slowly. Aerosols of various substituted phenols (125), hypochlorite mists (126), and other compounds (127, 128) have been studied abroad for such purposes. Most of the investigations in this country have been conducted by Robertson and various co-workers at the University of Chicago (129, 130, 131), who have worked mainly with triethylene glycol and propylene glycol. Quite a bit of controversy centered about such applications a few years ago (132, 133). In the main, the glycols did appear to lower air-borne bacterial counts, if the compounds were properly aerosolized in the right amounts, which was mechanically difficult. A more serious criticism of the treatment lies in the fact that no good epidemiologic data were gathered (134, 135, 136) to show that reducing the bacterial count of air in certain critical places such as barracks, public schools, and hospital wards had any appreciable effect in reducing the spread of air-borne infectious diseases.

Two possible reasons for this failure to control air-borne infections might be mentioned. The glycol vapors, while lowering the bacterial count of the air, did not have any appreciable effect on surfaces. As pointed out by Klarmann (137), streptococci and other organisms could be recovered in large numbers from blankets, floor dust, and other areas, and so could serve as secondary reservoirs. Another factor which could be mentioned is the difficulty of isolating test subjects, so that only treated air is being breathed. Even if the subjects themselves are not leaving treated areas, as in the case of hospital wards, outsiders are constantly coming in. Probably not until the air in all, or essentially all, public gathering places is treated, so that the test subjects and their contacts could not be receiving infections elsewhere, would we be able to tell whether or not air disinfection is effective in controlling respiratory infections. No such wide-scale applications of air disinfection have been made and the practice is essentially abandoned today, even in research studies.

## ETHYLENE OXIDE

It was not until ethylene oxide came into use that an effective, truly gaseous method of chemical sterilization became available. A decade ago no

textbook or review on chemical disinfectants mentioned this compound although Phillips & Kaye (138), in reviewing the subject in 1949 before publishing their first findings on its activity (139, 140, 141), found a dozen or more references to ethylene oxide's bactericidal activity in the literature, mostly obscure but including several patents.

The first series of publications by Phillips and Kaye dealt with the effects of time, concentration, temperature (139), and moisture (141) upon the bactericidal effect of ethylene oxide, using as a test agent the spores of *Bacillus subtilis* var. *niger* (*B. globigii*) which had been affixed to cotton patches. A third paper (140) dealt similarly with the effect of ethylene oxide upon aerosols of that organism. The most pertinent summation of these data is that at least four hours are required to sterilize highly contaminated materials at ambient relative humidities, room temperatures, and concentrations as high (442 mg./liter) as it is usually practical to reach. Thus the advantage of ethylene oxide sterilization does not lie in the speed with which it can be accomplished, nor for that matter in the simplicity or inexpensiveness of the treatment, but rather in the fact that many types of articles or materials previously considered impossible to sterilize without damage could now be safely treated.

A mechanism of action for ethylene oxide was proposed by Phillips (139) and expanded upon in a later paper (142). This mechanism was based upon the known alkylating action of ethylene oxide upon simple organic chemicals and proteins (143). In the presence of a labile hydrogen atom the unstable 3-membered ring of ethylene oxide splits. The hydrogen atom attaches itself to the oxygen, forming a hydroxy ethyl ($CH_2CH_2OH$) radical, which in turn attaches itself to the position in the organic molecule left vacant by the hydrogen. Fraenkel-Conrat (143) has shown that in proteins, carboxyl (COOH), amino ($NH_2$), sulfhydryl (SH), and hydroxyl or phenolic (OH) groups were each capable of supplying such a labile hydrogen. It was proposed that the replacement of this hydrogen by a hydroxy ethyl group would effectively block the reactive groups within bacterial proteins, hence causing an irreversible death as the substituted alkyl groups could be removed only by comparatively drastic chemical treatment.

Two other observations support this theory. Other compounds studied which were effective alkylating agents in organic chemistry were also effective bactericides. They were often gaseous ones, since most of these compounds were of low molecular weight and hence volatile. Besides ethylene oxide, the list of such alkylating compounds studied included formaldehyde, methyl bromide, and ethylene imine, which would add hydroxy methyl ($CH_2OH$), methyl ($CH_3$), and amino ethyl ($CH_2CH_2NH_2$) alkyl radicals, respectively, to reactive groups within organic molecules. A second observation which supported this proposed mechanism was that bacterial spores were relatively easy to kill with these alkylating compounds (142), being

only up to tenfold more resistant than vegetative bacteria. With other groups of disinfectants, such as oxidizing agents, heavy metals, phenols, and quaternary ammonium compounds, spores were several thousandfold more resistant than vegetative organisms. It was proposed that with the latter types of disinfectants, the principal group attacked was the sulfhydryl (SH), and that this group was less accessible to chemical action in spores than in vegetative cells. The alkylating agents, finding the sulfhydryl group blocked, however, could attack the other reactive groups referred to previously and hence disinfect through alternate pathways of action.

It should be noted that while this alkylating mechanism of action has not been challenged, earlier mechanisms had been proposed for at least two of these compounds. Gross & Dixon (144) in 1937 had claimed that the activity of ethylene oxide resulted from its conversion by hydrolysis to ethylene glycol, a known bactericide, but this had been contested by Yesair & Williams in 1942 (145) and by Wilson & Bruno in 1950 (146), who showed that the latter compound had a lesser bactericidal activity. Kolb & Schneiter in 1949 (147) had suggested that the bactericidal action of methyl bromide might be due to the fact that in its reaction hydrogen bromide was formed. This compound is released, however, only when alkylation has occurred to make the hydrogen available, and is released in very small amounts which would hardly account for the noted activity of methyl bromide. No such acids are formed with the other alkylating compounds which possess a similar and indeed more active bactericidal effect.

Ethylene oxide is apparently effective against all types of microorganisms. The earlier literature, containing references to its effect on many types of bacteria and molds, is covered in the review of Phillips & Kaye (138). Since then Ginsberg & Wilson (148) have reported on the activity of ethylene oxide solutions against several viral agents, and Mathews & Hofstad (149) on the effect of gas on 15 animal viruses, as well as several bacteria and fungi. Other viral agents recently reported to be sensitive to ethylene oxide include foot-and-mouth disease virus (150, 151), Columbia S. K. virus and vaccinia (152), and Eastern equine encephalomyelitis virus (153). Wilson & Bruno (146) and Newman *et al.* (154) reported on the sensitivity of *M. tuberculosis,* as did Kaye (155) for the BCG strain of that organism. Friedl, Ortenzio & Stuart (156) reported that five anaerobic and five aerobic spores were destroyed as measured by the Association of Official Agricultural Chemist sporicidal test. All indications to date are that the effect of ethylene oxide is irreversible, and bactericidal rather than bacteriostatic in nature.

Two distinct advantages of ethylene oxide over formaldehyde are its ability to penetrate porous materials and its lack of any requirement for excessive humidity in order to be effective. Phillips (157) reported that the gas would penetrate and kill spores through many layers of clothing and through unsealed paper wrapping. It would also penetrate and sterilize

through shallow layers of oil or water, and its action was not inhibited by the presence of such foreign material as raw eggs, feces, vaseline, and motor oil. Lorenz *et al.* (158) reported that the gas penetrated through unbroken egg shells, sterilizing the contents within. With vacuum treatment, penetration is achieved through whole barrels of spices. (159).

High humidities are not needed for ethylene oxide sterilization (141). As a matter of fact, sterilization is most easily accomplished at the usually encountered mid-range relative humidities. Some moisture, however, must be present since sterilization skips have been noted in high vacuum systems where the materials treated are extremely desiccated or essentially lyophilized.

A wide variety of equipment has been used for ethylene oxide treatment. The main requirement is that the equipment must be essentially hermetically sealed, since along with ethylene oxide's ability to penetrate goes the propensity for rapidly leaking out of any chamber not perfectly sealed. The Griffith & Hall patents (160 to 163) are based on treatment in large vacuum containers from which air is first removed and then pure ethylene oxide added. Kaye, Surkiewicz & Jacobson (164) reported on various types of improvised equipment including gas-tight bags and tarpaulins, in which the gas, either pure or diluted to prevent flammability, could be used without prior evacuation. Newman *et al.* (154) and Skeehan *et al.* (165) converted steam autoclaves into ethylene oxide exposure chambers. Especially developed equipment for ethylene oxide sterilization is now commercially available (166, 167).

A wide variety of materials or objects hitherto considered difficult or impossible to sterilize have been reported in the literature as successfully treated without damage by ethylene oxide. The largest number appeared in an article by Newman, Colwell & Jameson (154) who installed an ethylene oxide system to sterilize objects made in occupational therapy wards by patients in a tuberculosis hospital. Aside from all types of textiles, plastics, paper, and wood, their list even included such objects as oil paintings. Other materials reported as successfully sterilized included soil (168, 169, 170), laboratory plastics (171), medical and biologic preparations (160, 161, 162), hospital bedding and books (155), plaster of Paris bandages (172), various types of medical instruments (165, 173), and arteries for surgical transplants (174).

Not all materials, however, can be successfully treated without damage. Certain chemicals can be altered; for example penicillin can be successfully sterilized but a loss in potency occurs when streptomycin is treated (175). Mickelsen & co-workers (176, 177) showed that when certain whole animal diets were treated they were no longer capable of supporting life, a fact attributable to the destruction of certain essential vitamins. Damage to various types of plastic articles has recently been called to the authors' attention, but this has been thought to be attributable to the fact that the

sterilizing chambers were so constructed that objects came in contact with unvaporized liquid as it entered. The vapor itself does not appear to harm plastics.

Among the disadvantages noted for ethylene oxide, aside from its slowness of action, are its flammability and toxicity. The pure gas forms a flammable mixture in air in concentrations as low as three per cent. This can be avoided by dilution with inert gases such as carbon dioxide (178) or the fluorinated hydrocarbons (165). Ethylene oxide can now be obtained commercially in liquid form premixed with either of these diluents. This flammability safety is achieved only at a considerable loss in potency, since about 90 per cent of these mixtures is diluent.

The inhalation toxicity (179, 180, 181) is not too great, being about the same as ammonia gas, but cases have been reported in the literature of nonfatal accidents on this score (182, 183). A second toxic effect of ethylene oxide lies in the fact that its vapors are vesicant to skin if contact is prolonged. The pure liquid, if spilled on the skin, evaporates too rapidly to cause much damage, but laboratory workers' feet were badly blistered when they wore rubber shoes which had recently been sterilized (139). It was subsequently shown that ethylene oxide could remain as a solid solution in thick rubber for periods up to six hours, slowly releasing vapor during this period. Other industrial cases were reported (184, 185) in which workers' arms were burned after an aqueous solution of ethylene oxide had been spilled or splashed on their shirts. To avoid such accidents, any articles treated with ethylene oxide and intended for wear next to the skin, should be thoroughly aired, preferably for 24 hr. before donning.

## OTHER GASES

To any practical extent, the gases used in disinfection or sterilization are restricted to formaldehyde and ethylene oxide. Ozone, $O_3$, is bactericidal and has had some limited practical application (186, 187, 188) but is difficult to generate and handle, and moreover is damaging, particularly to rubber. Methyl bromide, $CH_3Br$, a widely used insecticidal fumigant, has already been mentioned as being also bactericidal (142, 147, 189). It has little advantage over ethylene oxide, however, is less active (122), and to date has had little if any practical use in disinfection. Chloropicrin, $Cl_3CNO_2$, has had some horticultural application as a soil fumigant, and was recently recommended for sterilization of ship compartments (189). Phillips (139) and Kaye (140) found that several chemicals structurally related to ethylene oxide also possessed bactericidal activity in the gaseous state; several, such as epichlorohydrin, epibromohydrin, ethylene sulfide, and particularly ethylene imine, were more active than ethylene oxide itself in this respect. Difficulty with commercial availability, among other things, however, has prevented their being put to practical use.

One very promising compound, β-propiolactone, has been extensively

studied in the authors' laboratory within the past few years, and a paper is now in press, co-authored by one of us (B. Warshowsky), summarizing its activity in the vapor state. The bactericidal and virucidal activity of this compound in aqueous solution has previously been reported by LoGrippo and co-workers (190, 191, 192). The present authors feel that the compound possesses almost all of the advantages of formaldehyde, and avoids two of its disadvantages, slow action and extreme persistency as a vapor, following use. It is believed that β-propiolactone should in the future replace formaldehyde in many applications where it is now used, and should find additional applications where the disadvantages of formaldehyde have limited its use.

## LITERATURE CITED


1. Wyss, D., *Ann. Rev. Microbiol.*, **2**, 413–34 (1948)
2. Geppert, J., *Berlin. klin. Wochschr.*, **26**, 789–94 (1889)
3. Kelner, A., *Proc. Natl. Acad. Sci. U. S.*, **35**, 73–79 (1949)
4. Kelner, A., *J. Bacteriol.*, **58**, 511–22 (1949)
5. Giese, A. C., Iverson, R. M., and Sanders, R. T., *J. Bacteriol.*, **74**, 271–79 (1957)
6. Heinmetz, F., Vinegar, R., and Taylor, W. W., *J. Gen. Physiol*, **36**, 207–26 (1952)
7. Heinmetz, F., *J. Bacteriol.*, **66**, 455–57 (1953)
8. Heinmetz, F., Lehman, J. J., Taylor, W. W., and Kathan, R. H., *J. Bacteriol.*, **67**, 511–22 (1954)
9. Heinmetz, F., Taylor, W. W., and Lehman, J. J., *J. Bacteriol.*, **67**, 5–12 (1954)
10. Garvie, E. I., *J. Appl. Bacteriol.*, **17**, XXI (1954)
11. Garvie, E. I., *J. Bacteriol.*, **69**, 393–98 (1954)
12. Chambers, C. W., Tabak, H. H., and Kabler, P. W., *J. Bacteriol.*, **73**, 77–84 (1957)
13. Chick, H. J., *J. Hyg.*, **8**, 92 (1908)
14. Meier, P., *Science*, **125**, 1067–71 (1957)
15. Francis, T., Jr., Korns, R. F., Voight, R. B., Boisen, M., Hemphill, F. M., Napier, J. A., Tolchinsky, E., *Am. J. Public Health*, **45**, 1–63 (1955)
16. Salk, J. E., Krech, U., Younger, J. S., Bennett, B. L., Lewis, L. J., and Bazeley, P. L., *Am. J. Public Health*, **44**, 563–70 (1954)
17. Salk, J. E., Lewis, L. J., Bennett, B. L., Ward, E. N., Krech, U., Younger, J. S., and Bazeley, P. L., *Am. J. Public Health*, **45**, 151–62 (1955)
18. Schwerdt, C. E., and Schaeffer, F. L., *Ann. N.Y. Acad. Sci.*, **61**, 822 (1955)
19. Dulbecco, R., and Vogt, M., *Ann. N.Y. Acad. Sci.*, **61**, 740 (1955)
20. Fogh, J., and Schwerdt, C. E., *Federation Proc.*, **15**, 253 (1956)
21. Reddish, G. F., Ed., *Antiseptics, Disinfectants, Fungicides, and Sterilization*, 2nd ed. (Lea and Febiger, Philadelphia, Pa., 975 pp. 1957)
22. Rideal, S., and Walker, J. T. A., *J. Roy. Sanit. Inst.*, **24**, 424–41 (1903)
23. *U. S. Food and Drug Administration Circ. No. 198* (1931)
24. *Official and Tentative Methods of Analysis*, 7th ed., 88–92 (J. Assoc. Official Agr. Chemists, 1950)
25. Morton, H. E., North, L. L., and Engley, F. B., Jr., *J. Am. Med. Assoc.*, **136**, 37-41 (1948)
26. Hunt, G. A., *J. Infectious Diseases*, **60**, 232 (1937)
27. Fildes, P., *Brit. J. Exptl. Pathol.*, **21**, 67–73 (1940)
28. Smith, D. C., Czarnetzky, E. J., and Mudd, S., *Am. J. Med. Sci.*, **192**, 790–808 (1938)
29. Engley, F. B., Jr., *Ann. N.Y. Acad. Sci.*, **53**, 197–206 (1950)
30. Brewer, J. H., *J. Am. Med. Assoc.*, **115**, 598–600 (1940)
31. Salle, A. J., and Ginoza, Y. W., *Proc. Soc. Exptl. Biol. Med.*, **54**, 85–87 (1943)
32. Duclaux, E., *Traite de microbiologie*, 461–503 Tome III (Masson et Cie, Paris, France, 1898)
33. Isaacs, M. L., *Science*, **75**, 46–48 (1932)
34. Stark, O. K., and Montgomery, M., *J. Bacteriol.*, **29**, 6–7 (1935)
35. Selzer, L., and Baumberger, J. P., *J. Cellular Comp. Physiol.*, **19**, 281–87 (1942)
36. Barron, E. S. G., and Singer, T. P., *J. Biol. Chem.*, **157**, 221–40 (1945)

37. Lamanna, C., and Mallette, M. F., *Basic Bacteriology* (Williams and Wilkins, Baltimore, Md., 677 pp. 1953)
38. Brewer, J. H., *J. Am. Med. Assoc.*, **137**, 858–61 (1948)
39. Powell, H. M., *J. Am. Med. Assoc.*, **137**, 862–66 (1948)
40. Cromwell, H. W., *J. Am. Med. Assoc.*, **140**, 401–3 (1949)
41. Brewer, J. H., and McLaughlin, C. B., *J. Am. Med. Assoc.*, **146**, 729–30 (1951)
42. Mortensen, J. D., Weed, L. A., and Grindlay, J. H., *J. Lab. Clin. Med.*, **44**, 604 (1954)
43. Davisson, E. O., Powell, H. M., MacFarlane, J. O., Hodgson, R., Stone, R. L., and Culbertson, C. G., *J. Lab. Clin. Med.*, **47**, 8–19 (1956)
44. Brewer, J. H., in *Antiseptics, Disinfectants, Fungicides, and Sterilization*, 2nd. ed., Chap. 12 (Reddish, G. F., Ed., Lea and Febiger, Philadelphia, Pa., 975 pp., 1957)
45. Sordelli, A., and Wernicke, R., *Compt. rend. soc. biol.*, **85**, 317 (1921)
46. Fischer, M., *Arch. Hyg.*, **94**, 215 (1924)
47. Herzberg, K., *Zentr. Bakteriol. Parasitenk.*, **90**, 113 (1923)
48. Degkwitz, R., *Klin. Wochschr.*, **8**, 342 (1929)
49. Von Plotho, O., *Biochem. Z.*, **110**, 1 (1920)
50. Bancroft, W. D., and Richter, G. H., *J. Phys. Chem.*, **35**, 511 (1931)
51. Fleming, A., and Young, M. Y., *J. Pathol. Bacteriol.*, **51**, 29 (1940)
52. Dunklin, E., *Chem. Specialties Mfrs. Assoc., Proc. 2nd Mid-year Meeting*, p. 110 (May, 1956)
53. Neisser, M., and Eichbaum, F., *Ergeb. Hyg. Bakteriol. Immunitätsforsch. u. Exptl. Therap.*, **13**, 170 (1932)
54. Goetz, A., *J. Am. Water Works Assoc.*, **35**, 579 (1943)
55. Kuroya, M., *Zentr, ges. Hyg. Bakteriol. u. Immunitäts.*, **14**, 565 (1927)
56. Krause, G. A., *Neue Wege zur Wassersterilisierung.* (J. F. Bergmann, Munich, Ger., 28 pp., 1928)
57. Guest, H. L., and Salle, A. J., *Proc. Soc. Exptl. Biol. Med.*, **51**, 272 (1942)
58. Goetz, A., Tracey, R. L., and Harris, F. A., Jr., *Silver in Industry*, 401–29 (Addicks, L., Ed., Reinhold Publ. Corp., New York, 636 pp., 1940)
59. Lieb, F., *Arch. Hyg.*, **116**, 317 (1936)
60. Fromberg, H., and Heiss, J., *Angew. Chem.*, **50**, 679 (1937)
61. Cade, A. R., *Proc. Sci. Sect. Toilet Goods Assoc.*, No. 7, 16–22 (1947)
62. Gesser, G. A., *Gas-u. Wasserfach*, **93**, 493–96 (1952)
63. Rochat, C., and Uzdins, K., *Schweiz. med. Wochschr.*, **77**, 1100–4 (1947)
64. Kliewe, H., Steyskal, K., and Steyskal, F., *Z. Hyg. Infektionskrankh.*, **127**, 110–22 (1947)
65. Laki, K., *Z. physiol. Chem. Hoppe-Seyler's*, **273**, 248 (1942)
66. Vande Velde, A. J. J., *Mededel. Koninkl. Vlaam. Acad. Wetenschap. Belg. Kl. Wetenschap.*, **9**, (12), 13 pp. (1947)
67. Ambrose, J. F., Kistiakowsky, G. B., and Kridl, A. G., *J. Am. Chem. Soc.*, **73**, 1231–36 (1951)
68. Yudkin, J., *Enzymologia*, **2**, 167 (1937)
69. Kruse, W., and Fischer, M., *Gesundh. Ingr.*, **59**, 312 (1936)
70. Konrich, F., *Gesundh. Ingr.*, **52**, 804 (1929)
71. Herrmann, A., *Schweiz. Apotheker-Ztg.*, **72**, 599 (1934)
72. Schwartz, G., *U.S. Pat. 2,459,896* (Jan., 1949)
73. Snell, F. D., and Shapiro, R. L., *U.S. Pat. 2,463,327* (March, 1949)

74. Barnes, R. B., and Ham, G. P., *U.S. Pat. 2,434,190* (Jan., 1948)
75. Banks, R. M., and Barnes, R. B., *U.S. Pat. 2,500,113* (March, 1950)
76. Quinn, D. H., *U.S. Pat. 2,595,290* (May, 1952)
77. Scheider, M., *Therap. Gegenwart.*, **90**, 421–23 (1951)
78. Hoffman, R. K., Surkiewicz, B. F., Chambers, L. A., and Phillips, C. R., *Ind. Eng. Chem.*, **45**, 2571–73 (1953)
79. Romans, I. B., in *Antiseptics, Disinfectants, Fungicides, and Sterilization*, 2nd ed., Chap. 21 (Reddish, G. F., Ed., Lean and Febiger, Philadelphia, Pa., 975 pp., 1957)
80. Mendenhall, E. E., *U.S. Pat. 2,510,510* (1950)
81. Goetz, A., *U.S. Pat. 2,521,713* (Sept., 1950)
82. Lindenmeyer, J., *German Pat. 807,123* (June, 1951)
83. Edlinger, E., *Ann. hyg. publ. ind. et sociale.*, **28**, 38–45 (1950)
84. Süpfle, K., and Werner, R., *Mikrochemie ver. Mikrochim. Acta*, **36/37**, 866–81 (1951)
85. Chambers, C. W., Chambers, L. A., and Kabler, P., *Ind. Eng. Chem.*, **45**, 2569–71 (1953)
86. Hotchkiss, R. D., *Ann. N.Y. Acad. Sci.*, **46**, 479–93 (1946)
87. Gale, E. F., and Taylor, E. S., *Nature*, **157**, 549–50 (1946)
88. Gale, E. F., and Taylor, E. S., *J. Gen. Microbiol.*, **1**, 77–84 (1947)
89. Salton, M. R. J., *Australian J. Sci. Research, Ser. B.*, **3**, 45–60 (1950)
90. Salton, M. R. J., *J. Gen. Microbiol.*, **5**, 391–404 (1951)
91. Newton, B. A., *J. Gen. Microbiol.*, **9**, 54–64 (1953)
92. Knox, W. E., Auerbach, V. H., Zarudnaya, K., and Spirites, M., *J. Bacteriol.*, **58**, 443–52 (1949)
93. Stedman, R. L., Kravitz, E., and King, J. D., *J. Bacteriol.*, **73**, 655–60 (1957)
94. Smith, C. R., Nishihara, H., Golden, F., Hoyt, A., Guss, C. O., and Kloetzel, M. C., *Public Health Repts. (U.S.)*, **48**, 1588–1600 (1950)
95. Lawrence, C. A., and Grover, A. A., *Bull. Am. Soc. Hosp. Pharmacists*, **8**, 224–28 (1951)
96. Toomey, J. A., and Takacs, W. S., *Arch. Pediat.*, **62**, 337–39 (1945)
97. Faber, H. K., and Dong, L., *Am. J. Diseases Children*, **86**, 469 (1953)
98. Shaughnessy, H. J., and Zichis, J., *Chronicle World Health Organization*, **10**, 805 (1954)
99. Alfredson, B. V., Stiefel, J. R., Thorp, F., Baten, W. D., and Gray, M. L., *J. Am. Pharm. Assoc., Sci. Ed.*, **40**, 263–67 (1951)
100. Woodward, G., and Calvery, H. O., *Proc. Sci. Sect. Toilet Goods Assoc.*, **3**, 1 (1945)
101. Krog, A. J., and Marshall, C. G., *Am. J. Public Health*, **30**, 343–47 (1940)
102. Mallman, W. L., Kivela, E., and Turner, G., *Soap Sanit. Chemicals*, **22**, 130 (1946)
103. Krog, A. J., and Marshall, C. G., *J. Milk Technol.*, **5**, 343–47 (1942)
104. Hucker, G. J., *Ann. Rept. N. Y. State Assoc. Milk Sanitarians, 21st Rept.*, 35–51 (1947)
105. Lawrence, C. A., *Surface-Active Quarternary Ammonium Germicides* (Academic Press, Inc., New York, N.Y., 240 pp., 1950)
106. Klarmann, E. G., and Wright, E. S., in *Antiseptics, Disinfectants, Fungicides, and Sterilization*, 2nd ed., Chap. 22 (Reddish, G. F., Ed., Lea and Febiger, Philadelphia, Pa., 975 pp., 1957)

107. Cade, A. R., and Gump, W. S., in *Antiseptics, Disinfectants, Fungicides, and Sterilization*, 2nd ed., Chap. 14 (Reddish, G. F., Ed., Lea and Febiger, Philadelphia, Pa., 975 pp., 1957)
108. Richardson, E. M., and Reid, E. E., *J. Am. Chem. Soc.*, **62**, 413–15 (1940)
109. Fogg, A. H., and Lodge, R. M., *Trans. Faraday Soc.*, **41**, 359–65 (1945)
110. Jordan, R. C., and Jacobs, S. E., *J. Hyg.*, **43**, 275–89 (1944)
111. Jordan, R. C., and Jacobs, S. E., *J. Hyg.*, **44**, 210–20 (1945)
112. Withell, E. R., *J. Hyg.*, **42**, 339–53 (1942)
113. Klarmann, E. G., and Shternov, V. A., *Ind. Eng. Chem. Anal. Ed.*, **8**, 369–72 (1936)
114. Maurice, P., *Proc. Soc. Appl. Bacteriol.*, **15**, 144–54 (1952)
115. Pulvertaft, R. J. V., and Lumb, G. D., *J. Hyg.*, **46**, 62–64 (1948)
116. Haydon, D. A., *Proc. Roy. Soc.* (*London*), [B]**145**, 383–91 (1956)
117. Bach, D., and Lambert, J., *Compt. rend. soc. biol.*, **126**, 298–302 (1937)
118. Sykes, G., *J. Hyg.*, **59**, 463–69 (1939)
119. Roberts, M. H., and Rahn, O., *J. Bacteriol.*, **52**, 639–44 (1946)
120. Hadfield, W. A., in *Antiseptics, Disinfectants, Fungicides, and Sterilization*, 2nd ed., Chap. 23 (Reddish, G. F., Ed., Lea and Febiger, Philadelphia, Pa., 975 pp., 1957)
121. Gershenfeld, L., in *Antiseptics, Disinfectants, Fungicides, and Sterilization*, 2nd ed., Chap. 11 (Reddish, G. F., Ed., Lea and Febiger, Philadelphia, Pa., 975 pp., 1957)
122. Phillips, C. R., in *Antiseptics, Disinfectants, Fungicides, and Sterilization*, 2nd ed., Chap. 30 (Reddish, G. F., Ed., Lea and Febiger, Philadelphia, Pa., 975 pp., 1957)
123. Nordgren, G., *Acta. Pathol. Microbiol. Scand.*, Suppl. 40, 1–165 (1939)
124. Salle, A. J., and Korzenovsky, M., *Proc. Soc. Exptl. Biol. Med.*, **50**, 12–16 (1942)
125. Mackay, I., *J. Hyg.*, **50**, 82 (1952)
126. Lidwell, O. M., and Lovelock, J. E., *Med. Research Council (Brit.) Mem. Spec. Rep. Ser. No. 262*, **68** (1948)
127. Lidwell, O. M., Lovelock, J. E., and Raymond, W. F., *Med. Research Council (Brit.) Mem. Spec. Rep. Ser. No. 262*, 82 (1948)
128. Raymond, W. F., *J. Hyg.*, **44**, 359 (1946)
129. Robertson, O. H., Bigg, E., Miller, B. F., and Baker, Z., *Science*, **93**, 213 (1941)
130. Robertson, O. H., Bigg, E., Puck, T. T., and Miller, B. F., *J. Exptl. Med.*, **75**, 593 (1942)
131. Robertson, O. H., Puck, T. T., Lemon, H. M., and Loosli, C. G., *Science*, **97**, 142 (1943)
132. Nagy, R., and Mouromseff, G., *Science*, **112**, 593 (1950)
133. Lester, W., Jr., Dunklin, E., and Robertson, O. H., *Science*, **115**, 379 (1952)
134. Report of Committee on Sanitary Engineering, National Research Council. *Am. J. Public Health*, **37**, 189 (1947)
135. Loosli, C. G., Smith, M. H. D., Gauld, R. L., Robertson, O. H., and Puck, T. T., *Am. J. Public Health*, **37**, 1385 (1947)
136. Personnel of U. S. Naval Medical Research Unit No. 4, *Am. J. Hyg.*, **55**, 203 (1952)
137. Klarmann, E. G., *Modern Sanitation*, **6**, (6) 15–18 (1954)
138. Phillips, C. R., and Kaye, S., *Am. J. Hyg.*, **50**, 270–79 (1949)

139. Phillips, C. R., *Am. J. Hyg.*, **50,** 280–89 (1949)
140. Kaye, S., *Am. J. Hyg.*, **50,** 289–95 (1949)
141. Kaye, S., and Phillips, C. R., *Am. J. Hyg.*, **50,** 296–306 (1949)
142. Phillips, C. R., *Bacteriol. Rev.*, **16,** 135–38 (1952)
143. Fraenkel-Conrat, H. L., *J. Biol. Chem.*, **154,** 227–38 (1944)
144. Gross, P. M., and Dixon, L. F., *U. S. Pat. 2,075,845* (April, 1937)
145. Yesair, J., and Williams, O. B., *Food Research*, **7,** 118–26 (1942)
146. Wilson, A. T., and Bruno, P., *J. Exptl. Med.*, **91,** 449–58 (1950)
147. Kolb, R. W., and Schneiter, R., *J. Bacteriol.*, **59,** 401–11 (1949)
148. Ginsberg, H. S., and Wilson, A. T., *Proc. Soc. Exptl. Biol. Med.*, **73,** 614–16 (1950)
149. Mathews, J., and Hofstad, M. S., *Cornell Vet.*, **43,** 452–61 (1953)
150. Savan, M., *Am. J. Vet. Research*, **16,** 158–59 (1955)
151. Callis, J. J., Tessler, J., Fellowes, O. N., and Poppensiek, G. C., *Bacteriol. Proc. (Soc. Am. Bacteriologists)*, **57,** 22 (1957)
152. Klarenbeek, A., and van Tongeren, H. A. E., *J. Hyg.*, **52,** 525–28 (1954)
153. Bucca, M. A., *J. Bacteriol.*, **71,** 491–92 (1956)
154. Newman, L. B., Colwell, C. A., and Jameson, E. L., *Am. Rev. Tuberc. Pulmonary Diseases*, **71,** 272–78 (1955)
155. Kaye, S., *J. Lab. Clin. Med.*, **35,** 823–28 (1950)
156. Friedl, J. L., Ortenzio, L. F., and Stuart, L. S., *J. Assoc. Offc. Agr. Chemists*, **39,** 480–83 (1956)
157. Phillips, C. R., *Bacteriol. Proc. (Soc. Am. Bacteriologists)*, 50th Meeting, 23–24 (1950)
158. Lorenz, F. W., Starr, P. B., and Bouthilet, R., *Poultry Sci.*, **29,** 545–47 (1950)
159. McCormick and Co., Baltimore, Md. (Personal communication, 1947)
160. Griffith, C. L., and Hall, L. A., *U. S. Pat. 2,189,947* (Feb., 1940)
161. Griffith, C. L., and Hall, L. A., *U. S. Pat. Re. 22,284* (March, 1943)
162. Griffith, C. L., and Hall, L. A., *U. S. Pat. 2,189,948* (Feb., 1940)
163. Griffith, C. L., and Hall, L. A., *U. S. Pat. 2,189,949* (Feb., 1940)
164. Kaye, S., Surkiewicz, B. F., and Jacobson, K. D., *Abst. Am. Chem. Soc.*, 122nd Meeting, 7s (1952)
165. Skeehan, R. A., Jr., King, J. H., and Kaye, S., *Am. J. Ophthalmol.* **42,** 424–30 (1956)
166. Perkins, J. J., *Principles and Methods of Sterilization*, 325–33 (Charles C Thomas Co., Springfield, Ill., 340 pp., 1956)
167. Kaye, S., and Darker, G. D., *Bacteriol. Proc. (Soc. Am. Bacteriologists)*, **57,** 22 (1957)
168. Clark, F. E., *Soil Sci.*, **70,** 345–49 (1950)
169. Roberts, J. L., Allison, L. E., Prickett, P. S., and Riddle, K. B., *J. Bacteriol.*, **45,** 40 (1943)
170. Allison, L. E., *Soil Sci.*, **72,** 341–52 (1951)
171. Engley, F. B., Jr., *Bacteriol. Proc. (Soc. Am. Bacteriologists)*, 51st Meeting, 22 (1951)
172. Wilson, A. T., *J. Am. Med. Assoc.*, **142,** 1067–68 (1950)
173. Bekker, J. H., and Onvlee, P. C., *Ned. Tjidschr. Genesk.*, **100,** 1491–95 (1956)
174. Hufnagel, C. A., Rabil, P. J., and Reed, L., *Surg. Forum, Proc. Congr. Am. Coll. Surgeons*, **4,** 162–68 (1953)
175. Kaye, S., Irminger, H. F., and Phillips, C. R., *J. Lab. Clin. Med.* **40,** 67–72 (1952)

176. Hawk, E. A., and Mickelsen, O., *Science*, **121**, 442–44 (1955)
177. Bakerman, H., Romine, M., Schricker, J. A., Takahashi, S. M., and Mickelsen, O., *J. Agr. Food Chem.*, **4**, 956–59 (1956)
178. Jones, G. W., and Kennedy, R. E., *Ind. Eng. Chem.*, **22**, 146–47 (1930)
179. Stehle, R. L., Bourne, W., and Lozinsky, E., *Arch. exptl. Pathol. Pharmakol., Naunyn-Schmiedeberg's*, **104**, 82–86 (1924)
180. Waite, C. P., Patty, F. A., and Yant, W. P., *Public Health Repts. (U.S.)*, **45**, 1832–43 (1930)
181. Greaves-Walker, W. J., and Greeson, C. E., *J. Hyg.*, **32**, 409–16 (1932)
182. Blackwood, J. D., and Erskine, E. B., *U. S. Naval Med. Bull.*, **36**, 44–45 (1938)
183. Anonymous, *Ind. Hyg. Newsletter*, **7**, (3), 6 (1947)
184. Sexton, R. J., and Henson, E. V., *J. Ind. Hyg. Toxicol.*, **31**, 297–300 (1949)
185. Sexton, R. J., and Henson, E. V., *Arch. Ind. Hyg. Occupational Med.*, **2**, 549–64 (1950)
186. Elford, W. S., and Van den Ende, J., *J. Hyg.*, **42**, 240–65 (1942)
187. Ingram, M., and Haines, R. B., *J. Hyg.*, **47**, 146–58 (1949)
188. Warshaw, L. J., *Am. J. Public Health*, **43**, 1558–62 (1953)
189. Saiki, E., *Nagoya J. Med. Sci.*, **15**, 270–76 (1952)
190. Szilagyi, D. E., Overhulse, P. R., Shonnard, C. P., and LoGrippo, G. A., *Surgical Forum, Proc. Congr. Am. Coll. Surgeons*, **5**, 244–52 (1954)
191. LoGrippo, G. A., Overhulse, P. R., Szilagyi, D. E., and Hartman, F. W., *Lab. Invest.*, **4**, 217–31 (1955)
192. Hartman, F. W., Kelly, A. R., and LoGrippo, G. A., *Gastroenterology*, **28**, 244–56 (1955)

# AUTHOR INDEX


A

Aaronson, S., 257, 289, 290
Abelson, P. H., 179, 184, 257
Abonnenc, C. y, 107
Abraham, R. G., 223, 226
Ackermann, W. W., 67
Adachi, R., 266
Adair, C. V., 51
Adams, S., 383
Addario, C., 114
Adelberg, E. A., 187, 316
Adelson, L. M., 272
Agar, H. D., 108
Ageeva, L. S., 401
Agosín, M., 108, 114
Airaper'ian, G. P., 403
Ajl, S. J., 180, 181, 182, 183, 249
Akiba, T., 339
Alcorn, S. M., 418, 421, 424, 430
Alexander, G. J., 263
Alexander, H. E., 326, 357
Alexander, J. K., 249
Alexander, M., 17, 229, 236
Alexopoulos, C. A., 375
Alfredson, B. V., 535
Aliev, A. N., 407
Alison, F., 50, 63
Aliverdiev, A. A., 401
Alleem, M. I. H., 229
Allen, E. J., 288
Allen, J. P., 369
Allen, M. B., 213, 215, 216, 235, 237, 289, 292, 302
Allen, O. N., 418, 430, 483
Allison, A. C., 141
Allison, F. E., 213
Allison, L. E., 542
Allison, R. M., 232
Al'tgauzen, A. J., 114
Altman, J., 425
Alves, W., 117
Alving, A. S., 111
Amano, T., 5, 8
Ambrose, J. F., 533
Amdur, B. H., 262
Ameel, D. J., 117
Ames, B. N., 252
Amies, C., 84
Ammich, O., 110
Amos, H., 270
Anchevs'ka, M. S., 404
Andersen, A. L., 420
Anderson, A. W., 514, 515
Anderson, E. H., 184, 296
Anderson, E. J., 444
Anderson, G. C., 286
Anderson, G. R., 237
Anderson, H. H., 106
Anderson, H. W., 416, 425, 431
Anderson, P. J., 427
Anderson, R. E., 165
Anderson, S. A., 51, 52, 59, 65
Anderson, T. F., 34, 340, 344
Anderson, W. P., 419, 420
Andonov, P. S., 392
Andrewes, C. H., 58, 65
Andrews, B. E., 49, 50, 58, 60, 61, 63, 64, 66, 67, 68, 69
Andrews, W. H. H., 111
Angel, H. R., 482
Angelo, C., 106
Ani, A. S. E., see El-Ani, A. S.
Ansari, N., 106
Anthony, D. S., 179, 183
Aposhian, H. V., 269
Apparao, A., 206
Appleman, M. D., 229
Apt, L., 113
Aquist, S., 164
Arber, W., 34, 35
Arie, T., 66
Ark, P. A., 415, 416, 418, 419, 421, 422, 430, 434
Arkhipova, O. P., 398
Armstrong, J. K., 453
Arndt, W. F., 5, 7, 11, 34, 346
Arnold, G., 111
Arnon, D. I., 222, 234, 235
Arnstein, H. R. V., 264
Arny, D. C., 428
Aronson, A. I., 15, 259
Aronson, B., 61, 64
Aronson, S., 61, 64
Artemova, L. I., 230
Artman, M., 204, 217
Asano, N., 267
Aschner, M., 292
Ash, J. E., 110
Ashby, D. R., 263
Askalonov, S. P., 397
Asnis, R. E., 209, 227
Assis-Lopes, L., 249
Astrachan, L., 41
Atkins, W. R. G., 287
Atkinson, D. E., 224, 250
Atkinson, H. J., 176
Atwood, K. C., 313, 373
Auclair, J. E., 267
Audus, L. J., 177
Auerbach, S. H., 50, 63
Auerbach, V. H., 535
Augier de Montgremier, H., 485
Aurand, L. W., 251
Averbach, B. C., 223, 226
Avigad, G., 164
Aytoun, R. S. C., 435
Azim, M. A., 115, 233, 234

B

Baalsrud, K., 213
Baalsrud, K. S., 213
Baar, H. S., 110
Babero, B. B., 117, 118
Bach, D., 536
Bach, M. K., 233, 299, 300
Bachrach, U., 184
Backus, M. P., 374, 375
Bacon, G. A., 78, 79, 87, 95, 96
Badger, G. F., 50, 51, 54, 55, 58, 59, 60, 61, 62, 63
Baer, H., 170
Baernstein, H. D., 103, 105
Bail, O., 81, 82, 87, 88, 91
Bailey, G. H., 81, 98
Bailey, L., 265
Baker, E. E., 81, 96
Baker, F., 160
Baker, H., 251, 289, 290
Baker, R., 375
Baker, Z., 539
Bakerman, H., 542
Bakerspigel, A., 373
Balch, H. W., 503
Bald, J. G., 442
Baldi, E., 293
Balducci, D., 58, 61
Baldwin, H. S., 503
Baldwin, I. L., 478
Baldwin, J. N., 504
Balis, M. E., 269
Ball, E., 477
Ball, G. H., 115
Ballamuth, W., 105
Bamforth, S. S., 282
Bancroft, W. D., 533
Bang, B., 138
Bang, F. B., 65, 66, 116
Banks, R. M., 534
Barber, M., 503, 504
Barbosa, F. S., 115, 116
Barbour, E., 367, 369
Barchielli, G., 253
Bard, R. C., 148, 154, 226
Barker, H. A., 146, 148, 152, 187, 188, 190, 212, 213, 216
Barksdale, W. L., 357
Barkulis, S. S., 180

Barner, H. D., 256, 259, 312, 313, 314, 339
Barnes, R. B., 534
Barnett, A. J. G., 223
Baron, L. S., 339, 347, 355
Barrington, L. F., 31, 34
Barron, E. S. G., 179, 531
Barski, G., 57
Bartmann, K., 18
Baten, W. D., 535
Bates, C. J., 510, 513
Batiuk, I. F., 404
Batt, R. D., 254
Batten, J. C., 502
Batzer, O. F., 508
Bauchop, T., 154
Bauman, N., 5, 9
Bauman, P. M., 116
Baumann-Grace, J. B., 4, 6
Baumberger, J. P., 531
Baumstark, J. S., 226
Bayley, S. T., 159
Bazeley, P. L., 528
Bazire, G. C., see Cohen-Bazire, G.
Beach, W. S., 426
Beadle, G. W., 365, 368, 370
Beal, R., 60
Beale, A. J., 50, 60, 61, 63, 67
Beale, G., 310
Beale, H. P., 416
Beattie, C. P., 109, 110
Beautyman, W., 113
Beaver, D. J., 270
Beaver, P. C., 104, 106, 112, 113, 114, 117
Bećarević, A., 351
Becker, C. E., 105
Becking, J. H., 237
Beecher, F. S., 422
Beer, M., 159
Behrer, M. R., 113
Behrman, E. J., 167
Beijerinck, M. W., 212, 443
Beiser, S. M., 332
Bekker, J. H., 542
Belaia, I. A., 397
Beletskaia, L. V., 396
Beliakov, E. V., 394
Beljanski, M., 261, 320
Beljanski, M., 261
Bell, J. A., 49, 50, 51, 52, 53, 54, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68
Bell, S. D., 63, 98
Bell, W., 422
Bellamy, W. D., 508, 510, 511, 512
Belozerskii, A. N., 388, 389
Belser, W. L., 344
Belt, M., 296
Belton, F. C., 92
Beltz, R. E., 270
Bendich, A., 332
Benenson, A. S., 116
Ben-Ishai, R., 259
Bennet, B. L., 528
Bennett, C. R., 56
Bennett, C. W., 442, 445, 446, 447, 448, 450, 451, 452
Bennett, M., 417
Benoit, R. J., 294, 301
Ben-Porat, T., 5, 15, 16
Bentler, E., 111
Bentley, R. L., 185
Benzer, 28
Benzer, S., 43, 348, 350, 369
Berberian, D., 106
Bergdoll, M. S., 494
Berge, T. O., 51, 58, 59, 63
Bergqvist, S., 500
Berk, R. S., 514, 515
Berkeley, G. H., 454, 455, 457, 462
Berman, L., 98
Bernasovs'ka, E. P., 404
Bernstein, H., 341, 358
Bernton, H. W., 51, 60, 61, 62
Bertani, G., 27, 44, 338
Bevacqua, R., 114
Beverley, J. K. A., 109
Bezborodov, A. M., 386
Bhattacharyya, P. K., 425
Bierstein, P., 117
Bietti, G. B., 62
Bigg, E., 539
Bilinski, E., 207
Billen, D., 512
Bird, T. J., 356
Bistis, G., 372, 376
Bitancourt, A. A., 473
Bitkova, A. N., 389
Black, L. M., 485
Black, W. A. P., 288
Blackwood, J. D., 543
Blagoveshchenskii, V. A., 391
Blair, J. E., 492, 493, 503
Blanchard, F. A., 418
Blattberg, B., 503
Blevins, A., 502
Bliuger, A. F., 397
Bloch, D. P., 56, 57
Bloch, H., 82, 86, 92, 93
Block, K., 262
Blom, N. O., see Oker-Blom, N.
Bloom, W. L., 82, 87, 88, 89, 90, 91
Bloomfield, R., 113
Blount, R. E., Jr., 49, 69
Blum, L., 111
Bobosch, K., 29, 30, 31, 34, 36
Bogoiavlenskaia, N. L., 392
Boisen, M., 528
Bolton, E. T., 179, 184, 257, 269
Bonanno, S., 263
Bonavia, L. L., 114
Bond, G., 237, 238, 291
Bonde, G. J., 250, 255
Bonde, R., 423, 424, 434
Bonner, D. M., 353, 368, 369
Bonner, J., 473
Bonnier, C., 236
Bonventre, P. F., 517
Boone, D. M., 372, 377
Boretti, G., 253
Borghans, J. G. A., 60
Borisova, M. A., 397
Boriu, S. I., 393
Borodinok, N. A., 396
Bostick, W. L., 106
Boswell, G. A., 263
Bourne, W., 543
Bouthilet, R., 542
Boutselis, J. G., 58
Bové, C., 234
Bové, J., 234, 474
Boyd, A. G., 141
Boyer, G. S., 54, 56, 57
Boyle, A. M., 415, 418
Boyle, J. S., 458
Boylen, J. B., 351
Bozicevich, J., 105
Braarud, T., 286, 295
Bracco, R. M., 326, 357
Bradin, J. L., Jr., 104, 105
Bradley, S. G., 346
Brady, F., 105
Braendle, D. H., 346, 358
Brainina, E. S., 401
Braithwaite, G. D., 262
Brakke, M. K., 485
Brandly, C. A., 77
Brandon, F. B., 52
Brasch, A., 508
Brase, K. D., 453, 454, 456, 459
Braun, A. C., 470, 477, 478, 479
Braun, W., 81, 97, 335
Brawerman, G., 258, 259
Breckoff, E., 61
Breed, R. S., 443
Brenes-Pomales, A., 366
Brenner, S., 6, 10, 15, 40, 350
Bresch, C., 42, 43
Brewer, J. H., 530, 531, 532
Brian, P. W., 415, 425, 431
Bridges, A. E., 514
Bridgmon, G. H., 461
Bridoux, M., 4, 10, 14
Brierley, P., 460, 461, 462
Brignon, J. J., 271
Bringmann, G., 18
Britt, E. M., 4, 7, 10
Britten, R., 205
Britten, R. J., 179, 184
Broadbent, D., 207
Broadbent, L., 448
Brodie, A. F., 152, 153, 236
Brogle, R. C., 520
Brooke, M. M., 103, 105, 106
Brooke, M. S., 186
Brooksby, J. B., 135

Brown, B., 187
Brown, D. D., 35
Brown, D. H., 163, 264
Brown, E. R., 325, 333
Brown, F., 301
Brown, G. B., 269
Brown, G. L., 333
Brown, G. M., 253
Brown, H. W., 105
Brown, I. B. R., 223
Brown, J., 110
Brown, J. G., 415, 417, 418
Brown, M. E., 237
Brownell, L. W., 227
Bruce White, G. B., 67
Bruemmer, J. H., 153, 236
Brug, S. L., 110
Brumpt, E., 103
Bruna, F., 62
Bruno, P., 541
Brutman, E. I., 387
Bucca, M. A., 541
Buchholtz, W. F., 457, 459
Buck, J. M., 139
Buckley, J. J. C., 117
Budovich, T., 165, 166
Budylina, V. V., 397
Budzhe, M. M., 397
Buffa, N. T., 514, 521
Bukrinskaia, A. G., 66
Bulen, W. A., 226, 234, 270
Bulmash, J., 251
Bundesen, H., 103
Bunting, M. I., 344
Burgi, E., 27, 39, 43
Burke, D., 419
Burke, J., 85, 86
Burkholder, L. M., 301
Burkholder, P. R., 301
Burma, D. P., 208, 232
Burnet, F. M., 65
Burnett, G., 444
Burnett, J. H., 376
Burns, V. W., 510
Burris, R. H., 203, 208, 230, 231, 232, 233, 234, 236, 481, 482, 486
Burrous, J. W., 346, 358
Burrows, T. W., 78, 79, 87, 95, 96
Burston, J., 504
Burton, K., 39
Burton, M. O., 265
Burton, R. M., 149
Bustamente, W., 110
Busvine, J. R., 112
Butler, J. A. V., 6, 10, 333, 365
Butler, R. L., 51, 58
Buttin, G., 259
Buxton, E. W., 378
Buyze, G., 149
Buzina, O. D., 230, 233

C

Cabib, E., 161
Cade, A. R., 533, 536, 537
Caglioti, M. T., 374
Cain, R. F., 513, 514, 515
Cairney, T. J., 421, 425
Caldwell, R., 113
Caldwell, R. M., 378
Calef, E., 369
Callely, A. G., 183
Callis, J. J., 541
Calvery, H. O., 535
Cameron, H. R., 455
Cameron, T. W. M., 117
Camien, M. N., 269, 271
Campbell, A., 324, 337
Campbell, J. J. R., 145, 181, 183, 184, 249
Campbell, L. L., Jr., 270
Campillo, A. del, 179
Candela, M. I., 221, 222
Cantino, E. C., 377
Cantoni, G. L., 258
Caplan, J. P., 113
Caplin, S. M., 474, 475
Cardini, C. E., 161, 264
Carey, W. F., 347
Carneiro, E., 116
Carpenter, C. M., 138
Carpenter, J. H., 290
Carr, M., 503
Carre, M. C., 57
Carrera, G. M., 103, 104, 113
Carritt, D. E., 290
Carson, S. F., 149, 179, 183
Carter, F. R. N., 103, 106
Carter, H. E., 425
Carvajal, F., 263
Case, M. E., 367, 369
Castex, M. R., 103
Catchside, D. G., 370
Cathie, I. A. B., 109
Cation, D., 417, 454, 455, 457
Cavalieri, L. F., 333
Cavalli-Sforza, L. L., 309, 341, 342
Ceglowski, W. S., 259
Chain, E., 502
Chaix, P., 264
Chamberlain, D. M., 109
Chamberlain, D. W., 429
Chamberlin, G. C., 457
Chambers, C. W., 527, 534
Chambers, L. A., 534
Chandler, A. C., 107
Chandler, V. L., 514, 521
Chang, E., 114
Chang, P. C. H., 107
Chang, R. S., 63
Chanock, R. M., 49-76; 49, 66, 67, 68, 69, 70
Chany, C., 50, 57, 63
Chapman, G. B., 1
Chaproniere, D. M., 54, 61, 65
Charalampous, F. C., 254
Chardome, M., 115
Chargaff, E., 5, 10, 19, 347
Charles, F. M., 419
Charm, S. E., 520
Charney, W., 263
Chase, M., 27, 30, 31, 37, 40, 42, 43
Cheever, F. S., 64
Cheldelin, V. H., 153, 154, 187, 267
Cheney, P. W., 457
Cheniae, G. M., 219
Chernomordik, A. B., 384
Chernysheva, M. I., 405
Cherry, W. B., 325
Chertkova, F. A., 403
Chertkova, M. A., 407
Cherviakova, K. I., 387
Chesbro, W. R., 514, 518
Chi, L., 68
Chichester, C. O., 262
Chick, H. J., 527
Chidester, J. L., 463
Chikaleva, L. V., 407
Chinn, B. D., 104
Chomwell, H. W., 532
Choporova, M. I., 387
Choquette, L. P. E., 103
Chow, A. W., 421, 425
Chow, T. J., 288
Christensen, J. J., 377
Chu, E., 324
Chu, G. W. T. C., 117
Chu, M. Y. W., 235
Chu, S. P., 283, 292, 294
Chumaevskaia, M. A., 398
Chung, C. W., 212, 215, 216, 225
Church, B., 146
Ciak, J., 5, 10, 18, 20, 259
Cifonelli, J. A., 163
Clark, F. E., 542
Clark, F. M., 254
Clark, J. H., 517
Clarke, N. A., 52
Clayman, C. B., 111
Clayton, C. N., 416, 457
Clayton, E. E., 425, 449
Cleveland, L. R., 104
Clifton, C. E., 187
Cline, J., 29
Clowes, R. C., 349
Clubb, M. E., 264
Coatney, G. R., 111
Cochran, G. W., 463
Cochran, L. C., 455, 457
Cockburn, T. A., 55, 60, 61, 64
Cockeram, A., 62
Cocking, E. C., 83, 95, 96, 97
Coe, D. M., 422, 424
Coelho, M. V., 116
Cohen, G. N., 193, 247, 258, 259, 268
Cohen, M. S., 181
Cohen, S. S., 256, 259, 312, 313, 314, 339

Cohen-Bazire, G., 356
Cohn, M., 146
Cole, C. R., 109
Cole, H., Jr., 428, 429, 430
Coleman, V. R., 51, 62
Collier, A., 287, 297
Collins, F. M., 213
Collins, H. S., 502
Collins, V. P., 518
Colobert, L., 4, 5, 6
Colovos, G. C., 508
Colvin, J. R., 158, 159
Colwell, C. A., 541, 542
Comegys, W. A., 425
Comolli, R., 340
Conover, R. A., 424, 425
Conover, S. A. M., 290
Conrat, H. L. F., see Fraenkel-Conrat, H. L.
Conway, E. J., 12
Cook, K., 49, 66, 67, 68, 69
Cook, M. K., 109
Cooley, L. M., 447
Cooper, D. C., 487
Cooper, P. D., 3, 20
Corey, R. R., 325
Cormier, M. J., 228
Cort, W. W., 117
Costa, A. S., 452
Costilow, R. N., 222, 251
Cothran, F. V., 320
Cotter, E. F., 50, 63
Couch, H. B., 428, 429
Coughlin, C. A., 316
Cowey, C. B., 299, 300
Cowie, D. B., 179, 184, 205, 257, 258
Cowper, S. G., 116
Cox, R. S., 421, 425
Craig, C. F., 105, 106
Cramblett, H., 67
Cramer, D. I., 108
Crandall, D. I., 167
Crane, F. L., 153, 236
Crane, H. R., 41
Crathorn, A. R., 6, 10
Crawford, I., 257
Crick, F. H. C., 38, 350
Cromartie, W. J., 82, 87, 88, 89, 90, 91, 94, 95
Crook, E. M., 160
Cropley, R., 457
Crosier, W., 428
Crossan, D. F., 421, 425
Crosse, J. E., 417
Crowdy, S. H., 432, 433
Cruikshank, R., 500
Crumpton, M. J., 79, 96
Cruz, E. E., 462
Culbertson, C. G., 106, 532
Cumming, C. S., 163
Cummins, C. S., 357
Cummins, J. T., 187
Curtice, C., 136
Curtis, O. F., 458
Curtis, R. W., 375
Curtiss, C., 54, 59, 60, 61, 62, 63
Cury, A., 289, 290
Cutchins, E. C., 53
Cuthbertson, W. F. J., 301
Cutinelli, C., 179
Cutter, V. M., 486
Cynkin, M. A., 149, 164
Czarnecki, H. J., 517
Czarnetzky, E. J., 530
Czosnowski, J., 480

D

Dack, G. M., 494, 520
Daengsvang, S., 114
Dagley, S., 167, 170, 171, 183
Daines, R. H., 417
Daisley, K. W., 299
Dark, F. A., 4, 9, 163
Darker, G. D., 542
Darling, H. M., 487
Darrow, G. M., 462
Dascomb, H. E., 51, 58, 59
Das Gupta, N. N., 108
Dashkevich, I. O., 406
Dauben, W. G., 263
Davidson, O. W., 429, 432
Davies, D. A. L., 79, 96, 166
Davies, D. L. G., 461
Davies, E. B., 235
Davis, B. D., 5, 9, 247, 268
Davis, B. H., 418, 425
Davis, C., 103
Davis, D., 68, 425
Davis, J. B., 179, 183
Davison, P. F., 365
Davison, S., 510
Davisson, E. O., 532
Dawes, E. A., 149, 151
Dawson, L. M., 3
De, M. L., 108
De, P. K., 291
Deane, L. M., 106
Deane, M. P., 106
DeBerry, P., 56
Deborin, G. A., 10, 14
De Busk, A. G., 365, 370
Dechary, J. M., 105
Decker, T. S., 16
Dedonder, R., 164
Deep, I. W., 419
DeFilippes, F. M., 334
de Freitas, J. F. T., see Teixeira de Freitas, J. F.
De Giovanni, R., 316, 334
Degkwitz, R., 533
De Groat, A., 113
de Haan, P. G., see Haan, P. G. de
Deibel, R., 62
Deibel, R. H., 271
Deicher, H., 335
Dekker, J., 423
DeLamater, J., 105
Delbrück, M., 27, 32, 38, 39, 42, 43, 321
Delbruck, M., 366
DeLeon, J. R., 107
DeLey, J., 151, 190
del Campillo, A., see Campillo, A. del,
del Ponte, E., see Ponte, E. del
Delwiche, C. C., 212, 213, 215, 216, 217, 223, 229
Delwiche, E. A., 145
De Magalhães, P. S., 114
Demain, A. L., 264
De Mars, R. I., 40
De Melo, J. L., 66, 67
Demerec, M., 318, 335 348, 349, 350
Demerec, Z., 335, 348, 350
de Montgremier, H. A., see Augier de Montgremier, H.
DeMoss, J. A., 184
DeMoss, R. D., 147, 148, 154
den Ende, J. V., see Van den Ende, J.
den Hamer, C. J. A. van, see Hamer, C. J. A. van den,
Dennis, E., 106
Denny, F. W., 94
Denny, F. W., Jr., 56
Dent, J. H., 113
Deotsch, R. N., 163, 166
der Meer, G Van, see Van der Meer, G.
Dern, R. J., 111
de Robichon-Szulmajster, H., see Robichon-Szulmajster, H. de
De Ropp, R. S., 81, 97
Derrick, E. H., 60
der Veen, J. van, see Veen, J. van der
de Serres, F., see Serres, F. de
de Serres, F. J., see Serres, F. J. de
Desjardins, P. R., 18
de Souza, P., see Souza, P. de
Desportes, C., 114
DeTurk, W. E., 204, 206
Dew, R. E., see Elsdon-Dew, R.,
Dewey, D. R., 509
de Zeeuw, D. J., see Zeeuw, D. J. de
Diachenko, S. S., 404
Diadichev, N. R., 395
D'iakov, S. I., 392
Dias, E., 107
Díaz, M., 110
Dickson, J. G., 377, 482

Didenko, S. I., 403
Dienes, L., 17, 18, 19
Di Marco, G. R., 418
Dimitrieva, N. P., 404
Dineen, P. A. P., 502
Dingle, J. H., 49, 50, 51, 54, 55, 58, 59, 60, 61, 62, 63, 497
Dixon, L. F., 541
Doane, F., 50, 60, 61, 63
Dobbins, J. E., Jr., 115
Doberti, A., 110
Dobrier, I. B., 397
Dobzhansky, T., 357
Docton, F. L., 109
Dodge, B. O., 376
Dodson, R. M., 263
Doermann, A. H., 27, 32, 40, 43
Domaradskii, I. V., 390
Dong, L., 535
Doolittle, S. P., 422, 425
Dorfman, A., 163
Dornbush, A. C., 267
Dorset, M., 142
Doty, D. M., 508, 518
Doudney, C. O., 315, 316, 511
Doudoroff, M., 147, 151, 184, 190
Douglas, H. C., 252, 369
Dove, W. E., 112
Dowding, E. S., 373
Dowler, A. M., 435
Downey, M., 12
Drabkin, D. L., 261
Draganov, K. I., 385
Dragys, J., 267
Drake, R. J., 452
Drake, S. D., 514, 518, 521
Drazin, R. S., 384
Dreisbach, A. R., 51
Driscoll, C. A., 257
Driver, C. H., 376
Droop, M. R., 284, 285, 286, 287, 289, 295, 296, 297 298, 299, 300
Drozhevkina, M. S., 406
Drysdale, R. B., 366
Dubini, 114
Dubos, R. J., 77, 78, 80, 81, 86, 93, 98, 503
Dubost, S., 271
Dubrovskaia, I. I., 404, 405
Duclaux, E., 531
Duffey, D., 509
Duggan, D., 514, 515
Duggar, B. M., 453, 470, 472, 478, 479, 480, 483
Duke, B. O. L., 115
Dulaney, E. L., 207
Dulbecco, R., 529
Dunaeva, T. N., 398
Duncan, C. W., 161
Dunegan, J. C., 416, 418
Dunklin, E., 533, 534, 539
Dunn, D. B., 258
Dunn, F. W., 268
Dunn, M. S., 269, 271
Dupetit, G., 211
Durham, N. N., 319
Durrell, M. E., 473
Dusi, H., 291, 296, 297
Duthie, E. S., 497, 502
Dutton, G. J., 161
du Vigneaud, V., see Vigeaud, V. du
Dworetezky, M., 503
Dye, D. W., 420
Dye, E. W., 417
Dye, M. H., 417, 434
Dzhikidze, E. K., 397

E

Eagles, B. A., 181, 183, 184, 249
Eaton, M. D., 70
Eaton, N. R., 184
Eberts, F. S., 481
Eddington, C. W., 511
Eddy, A. A., 21
Edlinger, E., 534
Edmondson, W. T., 286, 295
Efimova, N. P., 403
Efimova, V. A., 405
Egami, F., 209, 211, 215, 218, 221, 223, 226
Eggleston, L. V., 178
Egorova, S. V., 394
Ehrensvärd, G., 179
Eichbaum, F., 533
Eichhorn, A., 131
Eisenstark, A., 346
Eistrup, K., 267
Ekladius, L., 260
Ekstedt, R. D., 496
El-Ani, A. S., 375
Elberg, S. S., 86, 97
Elek, S. D., 493, 501
Elford, W. S., 543
Eliasson, N. A., 164
Elin, V. L., 389
Elion, G. B., 269
Ellenberger, C. E., 457
Ellis, D., 2
Elo, J. E., 2
El'piner, I. E., 387
Elsden, S. R., 145-202
Elsdon-Dew, R., 103
Elvidge, J. A., 168, 170
Elwyn, O., 186
Emel'ianova, O. S., 397, 405
Emerson, H., 417
Emerson, O. H., 415
Emerson, S., 365, 366
Enders, J. F., 49, 50, 51
Endo, Y., 265
Engalycheva, A. M., 387
Engel, M. S., 229
England, B., 51, 58, 59, 63
Engle, H. B., 426
Englehard, W. E., 247
Engler, J. I., 50, 51, 60, 61, 63, 64
Englesberg, E., 317, 335, 353, 354, 356
Engley, F. B., Jr., 530, 531, 532, 542
Enright, J. B., 142
Entner, N., 147
Ephrussi, B., 310
Ephrussi-Taylor, H., 333, 334
Epps, W. M., 423
Eppstein, S. H., 263
Ericson, L. E., 301
Erkama, J., 209
Ermachenko, V. A., 389
Errera, M., 314
Erskine, E. B., 543
Ertuganova, Z. A., 401
Esau, K., 451
Esposito, R. G., 235
Esser, K., 376
Eurich, E. W., 87, 88
Evans, A. H., 354
Evans, A. S., 60, 66, 116
Evans, D. G., 501
Evans, H. J., 205, 210, 218, 219, 221, 223, 225, 375
Evans, J. B., 271, 511, 513, 514, 517, 521
Evans, M., 110
Evans, W. C., 145, 167, 168, 171, 177, 178
Everitt, M. G., 103
Evseev, V. A., 407
Eyles, D. E., 109
Eyring, E. J., 187
Ezekiel, D. H., 15

F

Faber, H. K., 535
Faghih, M., 106
Fairley, J. L., 256
Fairley, N. H., 116
Falcone, G., 324
Fantuzzi, L. F., see Fischer-Fantuzzi, L.
Farkas-Himsley, H., 204, 217
Farmer, V. C., 174
Faulkner, L. R., 487
Faust, E. C., 103-26; 103, 104, 105, 106, 107, 111, 112, 113, 114, 115, 117
Fawcett, H. S., 442
Fedorov, M. V., 213, 225, 234, 389, 394
Fedotina, V. L., 384
Feingold, D. S., 164
Felber, I. M., 420
Feldman, H. A., 109
Fellowes, O. N., 541
Felton, L. D., 81, 98
Feng, L. C., 114
Fenn, W. O., 320
Fergus, C. L., 430
Few, A. V., 4, 7

Field, M. F., 267
Fildes, P., 9, 530
Files, V. S., 116
Filfus, J., 298
Filippovich, E. M., 387
Finberg, L., 69, 70
Fincham, J. R. S., 351, 369, 370
Fink, H. C., 457, 458
Fink, K., 256
Fink, R. M., 256
Finkelstein, R. A., 266
Fischer, A., 2, 4
Fischer. G. W., 377
Fischer, M., 533
Fischer-Fantuzzi, L., 338
Fisher, A. M.. 496, 499, 500
Fisher, E., 229
Fisher, E. G., 221, 222
Fisher, H. H., 415, 419
Fisher, K. W., 184, 185, 345
Fisher, T., 229
Fisher, T. N., 56
Fitzgerald, G. P., 283, 285
Fjerdingstad, E., 281, 282
Fleming, A., 533
Fling, M., 352, 370
Flock, H., 107
Flor, H. H., 377
Florentin, A., 114
Floyd, J., 94
Foard, M., 66
Fogg, A. H., 536
Fogg, G. E., 206, 291, 301, 302
Fogh, J., 529
Foghammar, S., 281
Folkes, J. P., 247, 248
Folsom, D., 443
Fonken, G. J., 263
Ford, J. E., 252, 253, 300
Ford, J. H., 417
Formal, S. B., 339
Forro, F., Jr., 323
Forssell, P., 55, 60, 61, 64
Forsyth, P. J., 56
Foster, F. L., 509
Foster, J. W., 10, 179, 183, 192, 259, 260
Foster, L. E., 81, 96
Foster, S. M., 149, 151
Fowle, A. M. C., 62
Fox, M. S., 317, 328, 330, 333
Fox, V. L., 55
Fraenkel-Conrat, H. L., 540
Francis, T., Jr., 49, 50, 51, 528
Franke, W., 181
Franklin, A. L., 296
Franklin, R., 78
Fraser, D., 5, 6, 8, 11, 34, 35, 347
Fraser, M. J., 4, 7
Fraunce, F., 97
Frazier, N. W., 462
Frear, D. S., 226, 234, 270
Freeman, B. M., 504
Freidman, M., 56, 58
Freitas, J. F. T. de, see Teixeira de Freitas, J. F.
French, D., 164
French, R. C., 270
Frenkel, J. F., 110
Freundt, E. A., 18
Fridlund, P. R., 459
Fried, J., 263
Friedl, J. L., 541
Friend, J. E., 433, 434
Fromberg, H., 533
Frye, W. W., 103, 105, 106
Fuchs, A. R., 250, 255
Fuerst, C. R., 33, 38, 322, 344
Fujikawa, K., 5, 8
Fujiwara, A., 222
Fukai, K., 67
Fukui, G. M., 95
Fukumi, H., 66
Fuld, G. J., 512, 513
Fülleborn, F., 112, 113
Fuller, R., 84, 97
Fulmer, E. I., 267
Fulton, J. D., 108
Fulton, R. W., 457, 459, 460
Furness, G., 341, 342, 358
Fusillo, M. H., 499

G

Gaby, W. L., 262
Gaden, E. J., Jr., 518, 521
Gafford, R. D., 167, 168, 170
Gaither, N., 314
Galaev, I. V., 388, 391
Gale, A. J., 509
Gale, E. F., 247, 248, 258, 260, 535, 536
Gallicchio, V., 117
Gallop, R. C., 79, 87, 88, 90, 91, 92
Galloway, B., 162
Gal'perin, E. A., 399
Gambogi, P., 431
Ganapathi, K., 264
Ganzburg, S. E., 399
Garber, E. D., 78
Garbowski, L., 2
Garcia-Laverde, A., 114
Gardash'ian, A. M., 403
Gardner, F. E., 453
Gardner, I. C., 237
Gardner, P. S., 66, 67
Garen, A., 29, 42, 344
Garner, G. B., 226
Garnham, P. C. C., 108, 111
Garnjobst, L., 372
Garvie, E. I., 527
Gary, N. D., 165
Gattani, M. L., 429
Gauld, R. L., 51, 52, 59, 65,539
Gäumann, E., 302
Gausmanova, I., 405
Gautheret, R. J., 472, 473, 484
Gauze, G. F., 393, 394
Gavin, J. J., 271
Gavrilova, L. P., 388
Gayon, V., 211
Gedz', S. M., 398
Geiman, Q. M., 105
Gekker, V. D., 397
Gelman, N. S., 4, 7, 10, 14, 390
Genny, A. T., 493
Gentles, M. J., 263
Georgi, C. E., 16, 165, 166
Georgopoulos, G., 112
Geppert, J., 526, 531
Gerhardt, P., 3, 4, 6, 7, 10, 17
Gerloff, G. C., 283, 285, 300
Gerngross, O. B., 66
Gerschman, R., 320
Gershenfeld, L., 537
Gershenzon, S. M., 384, 385
Gerth, R., 110
Gesser, G. A., 533
Gest, H., 148, 188, 189, 230, 231, 233
Ghiretti, F., 179
Ghose, N., 114
Gibbs, M., 147, 148, 149, 164
Gibbs, M. H., 262
Gibor, A., 296
Gibson, C. L., 109
Giddings, N. J., 451
Giese, A. C., 526
Giese, A. M., 314
Gilbert, D. L., 320
Gilby, A. R., 4, 7
Gilder, H., 210
Giles, N., 367, 369
Giles, N. H., 367, 369, 374
Giles, N. H., Jr., 319, 320, 352
Gilmer, R. M., 417, 455, 457, 459
Gilmore, L. K., 50, 53, 64
Ginoza, Y. W., 530
Ginsberg, A., 148
Ginsberg, H. S., 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 541
Ginzburg, T. S., 393
Girard, G., 96, 97
Girbardt, N., 370
Gladstone, G. P., 9, 92
Glander, R. von, 61, 64
Glaser, L., 158, 159, 162, 163, 264
Glasgow, H., 456
Glass, B., 204, 365, 368
Glenn, J. L., 153, 236
Glick, M. C., 209
Glover, J., 263
Glover, S. W., 318
Gloyer, W. O., 456
Gnevysheva, E. F., 405

Gnorizova, V. M., 66
Godman, G. C., 56, 57, 58
Goebel, W. F., 29, 35
Goetz, A., 533, 534
Gold, A. M., 263
Gold, E., 50, 53, 54, 59, 60, 61, 62, 63
Gold, K., 297
Goldberg, E. D., 288
Goldberg, M. K., 253
Goldblith, S. A., 508, 509, 510, 512, 513, 517, 518, 519, 521, 522
Golden, F., 535
Goldkamp, A. H., 263
Goldman, M., 105
Gol'farb, D. M., 406
Gollub, E. G., 355
Golueke, C. G., 250
Gomberg, H. J., 520
Gonin, S. L., 403
Gönnert, R., 116
Gonzalez, J. O., see Oliver-Gonzalez, J.
Goodgal, S. H., 320, 327, 330, 333, 335
Goodman, R. N., 416, 417, 432, 435
Goodpasture, E. W., 50, 63
Goodwin, T. W., 254, 262
Goos, J. J. C., 212
Gopalkrishnan, K. S., 425
Gorbunova, A. S., 66
Gordienko, A. N., 407, 408
Gordon, H. A., 103
Gordon, M. A., 270, 325
Gordon, R. M., 115
Gorini, L., 261
Gortner, R. A., 442
Goto, O., 265
Gots, J. S., 269, 355, 356
Gottlieb, D., 416, 425
Gould, J. C., 503
Gould, S. E., 520
Gowdridge, B. M., 373
Grabar, P., 383-414
Grabowich, P., 263
Grace, J. B. B., see Baumann-Grace, J. B.
Graham, A. F., 32
Graikoski, J. T., 517
Grainge, E. B., 108
Granick, S., 210
Gray, C.T., 83, 86,152, 153,236
Gray, M. L., 141, 535
Gray, P. H., 173
Gray, R., 417
Gray, R. A., 420, 431, 433
Grayston, J. T., 53, 54, 55, 58, 60
Greathouse, G. A., 156, 157, 158, 159, 264
Greaves-Walker, W. J., 543
Green, H. N., 95
Green, J. A., 353
Green, M., 256
Green, S., 334
Greenberg, J., 104
Greene, R. C., 268
Greenfield, S. S., 287
Greenspan, G., 263
Greenway, D., 103
Greer, S., 334
Greeson, C. E., 543
Gregory, K., 430
Gregory, M. E., 252
Griffith, C. L., 542
Griffith, J. S., 350
Griffiths, M., 356
Grimes, R. W., 161
Grindlay, J. H., 532
Groman, N. B., 339
Gromet, Z., 157, 158, 159, 160
Groot, H., 107
Gross, P. M., 541
Gross, S. R., 167, 168, 170, 356, 365, 370
Grossman, E. J., 106
Grosso, J., 426
Grosso, J. J., 426
Groth, D. P., 13
Grove, J. F., 433
Grover, A. A., 535
Grubeshova, M., 402
Grummer, G., 421
Gubarev, E. M., 388, 391
Guengerich, H. W., 462
Guest, H. L., 533
Guex-Holzer, S., 2, 3, 6, 11
Guha, A., 108
Guild, W. R., 334
Guirard, B. M., 247-78
Gump, W. S., 536, 537
Gundersen, K., 228, 229
Gunsalus, I. C., 147, 148, 150, 154, 164, 181, 183, 249
Gunter, S. E., 510, 511
Gurevich, R. M., 398
Gurin, S., 178
Gurvich, A. E., 402, 406
Guss, C. O., 535
Gustafson, P. V., 108
Guthrie, R., 272
Gutsche, A., 480
Gylybov, K. C., 405
György, P., 113, 254

H

Haan, P. G. de, 149
Haas, F. L., 315, 316
Hachisuka, Y., 267
Hacker, R. G., 428
Hackett, J., 78
Hadfield, W. A., 537
Hadley, C., 262
Hagan, W. A., 127-44
Hagborg, W. A. F., 428
Häggman, J., 209
Hahn, E., 326, 334, 357
Hahn, F. E., 5, 10, 18, 20, 259
Haines, R. B., 543
Hakala, M. T., 252
Hakansson, E. G., 103
Haldane, J. B. S., 370
Hale, J. H., 495, 496
Hales, N. B., 499
Hall, L. A., 542
Hall, L. M., 207
Hall, N. S., 219
Hall, R. P., 289
Halliwell, G., 160, 161
Hallman, F. A., 105, 492
Hallman, N., 110
Halse, G. R., 293
Halvorson, H. O., 146, 270
Ham, G. P., 534
Hamer, C. J. A. van den, 149
Hamilton, J. M., 417, 418
Hamilton, P. B., 230, 231, 237
Hamlin, A., 52, 56, 57
Hammarsten, E., 164
Hammer, C. L., 420
Hammerle, O. A., 513
Hampton, J. E., 418
Hanabusa, J., 62, 65
Hanby, W. E., 81
Hanks, J. H., 83, 86
Hanna, L., 51, 61, 62, 63
Hannan, R. S., 508, 509, 520
Hanotier, J., 4, 10, 14
Hansen, O. H., see Holm-Hansen, O.
Hansen, R. W., 212
Hanson, E. D., 310
Hantover, M. J., 51, 65
Happold, F. C., 170, 183
Harboe, A., 109
Hardin, G., 303
Hardwick, W. A., 10
Hardy, A. V., 103
Harein, P. K., 165
Harford, C. G., 52, 56, 57
Harinasuta, C., 104
Harnack, G. A., 61, 64
Harper, E. M., 162
Harper, G. J., 493
Harrar, J. G., 377
Harray, I., 167
Harris, C. H. S., see Stuart-Harris, C. H.
Harris, F. A., Jr., 533
Harris, H., 163, 357
Harris, M., 156, 157
Harris, R. V., 462
Harris, S. T., 160
Harris-Smith, P. W., 78, 79, 82, 84, 85, 88, 89, 90, 91, 92, 93
Hart, H., 427
Hartley, J. W., 51, 52, 53, 55, 56, 61, 65
Hartman, F. W., 544
Hartman, P. E., 153, 236, 324, 348, 349, 350
Hartman, R. E., 258
Hartman, Z., 319, 346, 350
Harvey, H. W., 212, 286, 288, 290, 294

Hashida, W., 265
Hashimoto, K., 327
Hashimoto, Y., 301
Haskins, C. P., 288
Hassid, W. Z., 148
Hattori, A., 223
Hauer, A., 106
Hauge, J. G., 189
Hauser, G., 166, 186, 264
Havlík, O., 109
Hawk, E. A., 542
Hawthorne, D. C., 372
Haxo, F. T., 297
Hayaishi, O., 167, 171, 190, 213, 260
Hayashi, S., 66, 67
Hayes, W., 184, 340, 341, 343, 345
Heath, E. C., 148, 166
Heckly, R. J., 82, 88, 89, 90, 91
Hedberg, M. A., 92
Heep, D. M., 415, 417
Heggested, H. E., 425, 426
Heilbrunn, I., 103
Heinmetz, F., 527
Heinrich, H. C., 298
Heiss, J., 533
Hellbrugge, T., 109
Helper, A., 502
Hemming, H. G., 415
Hemphill, D. D., 432
Hemphill, F. M., 528
Henderson, J. H. M., 473
Henderson, K., 35
Henderson, M. E. K., 174, 176
Henderson, R. B., 256
Henderson, R. G., 448
Hendricks, J. R., 117
Henis, D. B., 262
Henis, Y., 164
Henley, E. J., 518, 521
Henning, M. W., 130
Henricksson, E., 291
Henry, A. W., 428
Henson, E. V., 543
Heppel, L. A., 239
Herick, W. van, 70
Hernandez, M. G., 191
Hernández, T., 111
Herrer, A., 106
Herriott, R. M., 27, 35, 320, 327, 330, 333, 335
Herrmann, A., 534
Herrmann, R. L., 256
Hershberg, E. B., 263
Hershey, A. D., 27, 30, 31 36, 37, 38, 39, 41, 42, 43, 44
Hervey, A., 297, 301, 471, 486
Hervey, G. E. R., 422
Herzberg, K., 533
Herzog, H. L., 263
Hess, V. F., 258
Hestrin, S., 155, 156, 157, 158, 159, 160, 164
Heuberger, J. W., 416, 421, 425
Heussleman, B., 267
Hewitt, E. J., 221, 222
Hewlett, R. T., 87, 88
Heyl, J. T., 70
Heym, G. A., 187
Heyningen, W. E. van, 98
Hezebicks, M. M., 497
Hiai, S., 231
Hickman, M. L. W., 103
Hidaka, Z., 434
Higgins, E. S., 222, 228
Hijmans, M. D., 18
Hildebrand, E. M., 441-68; 448, 449, 454, 455, 456, 457, 458, 463
Hildebrandt, A. C., 469-90; 470, 472, 476, 477, 479, 480, 481, 482, 483, 485, 486, 487
Hildreth, R., 419
Hildreth, R. C., 419
Hilker, D. M., 105
Hillary, E. B., 249
Hillary, E. P., 181, 183
Hilleman, M. R., 49, 50, 51, 52, 53, 55, 58, 59, 63, 64, 65
Hillier, J., 1, 18
Hillman, M. J., 272
Hills, G. M., 270
Himsley, H. F., see Farkas-Himsley, H.
Hinchliffe, M. C., 117
Hino, S., 231, 234
Hirasawa, N., 67
Hirota, Y., 342
Hirsch, J. G., 8
Hirsch, M. L., 268
Hirth, L., 485
Hitchborn, J. H., 459
Hitchcook, D. J., 103
Hitchens, A. P., 443
Hitchings, G. H., 269
Hitzman, D. O., 270
Hoagland, M. B., 253
Hoare, C. A., 103, 106, 107
Hoare, D. S., 5, 9, 10, 271
Hobart, O. F., 457, 459
Hoch, G. E., 231
Hockey, J. F., 462
Hockwald, R. S., 111
Hodgson, B., 183
Hodgson, R., 532
Hodgson, W. A., 424
Hoeft, G. L., 106
Hoekenga, M., 106
Hoff-Jorgensen, E., 267, 272
Hoffman, R. K., 534
Hoffman, T., 229
Hoffmann, C. E., 267, 296
Hofmann, K., 262
Hofmeyer, J. D. J., 452
Hofstad, M. S., 541
Hogan, M. J., 64
Hogstrom, G., 179
Holbert, J. R., 482
Holden, J. T., 271
Holdsworth, E. S., 252, 253
Hollaender, A., 511, 512
Holliday, R., 375
Holloway, B. W., 372
Holman, H. R., 335
Holman, J., 271
Holme, T., 164, 165
Holmes, F. O., 442, 443, 448, 450
Holmes, F. W., 430
Holm-Hansen, O., 300
Holt, S. J., 86
Holton, C. S., 377
Holzaepfel, J. H., 58
Holzer, S. G., see Guex-Holzer, S.
Hope Simpson, R. E., 67
Höpken, W., 18
Hoppert, C. A., 161
Hopps, H. E., 259
Horecker, B. L., 146, 148, 184
Horner, C. E., 429, 435
Horowitz, N. H., 352, 370
Horsfall, F. L., 70
Horvath, A., 269
Hosoda, J., 8
Hotchkiss, R. D., 312, 326, 327, 328, 330, 331, 332, 354, 535
Hotson, H. H., 486
Houck, U. G., 130
Housain, I., 250
Houwink, A. L., 1
Hovenkamp, H. G., 153, 236
Howard, B. H., 163, 166
Howard, F. L., 428
Howard, J. G., 504
Howard, R. B., 165
Howe, C., 52, 56, 57, 58
Howe, H. B., Jr., 371
Howie, J. W., 77
Hoyem, H. M., 105
Hoyt, A., 535
Hrenoff, A. K., 104
Hsiang, Y. W., 221
Hsü, H. F., 115, 116, 117
Hsü, S. Y. Li, 116
Huang, J. S., 58
Hubendick, B., 117
Huber, O., 18
Huber, W., 508
Hucker, G. J., 535
Huddleson, F. I., 97
Huebner, R. J., 49-76; 49, 50, 51, 52, 53, 54, 55, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69
Huertos, M. R., see Rubio Huertos, M.
Huff, J. W., 262
Hufnagel, C. A., 542
Hug, D. H., 189
Hughes, D. T., 375
Hughes, J. P., 366

Hughes, W. A., 461
Hughes, W. H., 270
Hughes, W. L., 321
Hughes, W. L., Jr., 323
Hull, R. N., 51, 56
Human, M. L., 338
Humgate, R. E., 160
Humphreys, T. W., 222, 251
Hunt, G. A., 530
Hunter, G. D., 6, 10
Hunter, G. W., III, 112, 113, 117
Huppert, J., 5, 8
Hurst, R. R., 423
Hurwitz, J., 148
Hussey, K. L., 105
Hutchings, B. L., 267
Hutchins, L. M., 458
Hutchinson, F., 510
Hutchinson, G. E., 290, 292, 300
Hutner, S. H., 251, 253, 285, 288, 289, 290, 296, 297, 298, 299, 300, 310
Hyadon, D. A., 536
Hyatt, M. T., 270
Hyre, R. A., 421

I

Iakhno, M. A., 405
Iamshchikov, V. P., 386
Ichihashi, Y., 66
Ichikawa, S., 5, 8
Ichioka, P. S., 222
Iino, T., 355, 356
Ilina, T. K., 225
Imamaliev, S. A., 399
Imboden, C. A., 106
Imshenetskii, A. A., 230, 233
Ingraham, J. L., 167, 168
Ingraham, L., 356
Ingram, M., 543
Inogamov, A. B., 387
Ioffe, V. I., 403
Irminger, H. F., 542
Isaacs, M. L., 531
Ishai, R. B., see Ben-Ishai, R.
Ishida, N., 66, 67
Ishihara, S. J., 421, 425
Ishikawa, T., 375
Ishimoto, M., 227
Ishitani, C., 374
Ispolatovskaia, M. V., 404
Iterson, W. van, 1
Ito, N., 66
Iurkov, I. A. 396
Iverson, R. M., 314, 526
Iwanowski, D., 443
Iwasaki, H., 217

J

Jaag, O., 302
Jachowski, L. A., 115
Jachowski, L. A., Jr., 115
Jackson, A. W., 495
Jackson, L. W. R., 442
Jacob, F., 27, 33, 42, 43, 44, 45, 337, 338, 340, 341, 342, 343, 344
Jacobs, L., 104, 108, 109, 110
Jacobs, S. E., 536
Jacobson, K. D., 542
Jacoby, J., 114
Jacquiot, C., 487
Jaffurs, W. J., 499
Jagger, J., 320
Jakob, H., 302
Jakoby, W. B., 190
James, A. P., 373, 374
Jameson, E. L., 541, 542
Janssen, W. A., 95
Jansson, E., 55
Jarvis, F. G., 166
Jawetz, E., 51, 61, 62, 63, 79
Jaysuriya, G. C. N., 184
Jebb, W. H. H., 251, 265
Jefferson, W. E., 179, 183
Jeffery, G. M., 111
Jenkins, A. R., 108
Jensen, A. D. O., see Orla-Jensen, A. D.
Jensen, H. L., 177, 228, 255
Jensen, J., 261
Jensen, K. A., 319
Jensen, K. E., 67
Jensen, S. O., see Orla-Jensen, S.
Jensen, V., 237
Jerne, N. K., 39
Jesaitis, M. A., 29, 35
Jeynes, M. H., 5, 11
Jinks, L. L., 342
Jirovek, O., 110
Jo, K., 66
Joe, L. K., see Lie, Kian Joe
Johann, H., 482
Johns, A. T., 179
Johnson, J., 443, 453
Johnson, M. J., 20, 166
Johnson, R. E., 98
Johnson, V. M., 114
Johnson, W. H., 296, 299
Johnston, P. B., 54, 55, 60
Johnston, R., 301
Johnstone, H. G., 106
Jones, E. S., 111
Jones, F., 105
Jones, F. E., 109
Jones, G. W., 543
Jones, J., 114
Jones, J. D., 171
Jones, L. K., 444
Jones, M. E., 181, 301
Jones, M. F., 115
Jones, W. R., 104
Jordan, E. M., 33, 35
Jordan, R. C., 536
Jordan, W. S., Jr., 50, 51, 53, 54, 55, 58, 59, 60, 61, 62, 63, 98
Jordon, R. T., 519
Jørgensen, E. H., see Hoff-Jørgensen, E.
Jose, A. G., 232
Josephson, E. S., 104
Joslin, R. P., 520
Josten, J. J., 236
Josten, J. L., 153
Judd, A. W., 141
Judis, J., 230, 231, 233
Jukes, T. H., 267, 296
Juliano, S., 114
Julita, P., 253
Jump, J. A., 425
Jung, R. C., 104, 106
Jungherr, E., 66
Juni, E., 137
Ju-Shan, L., see Lu Ju-Shan

K

Kabler, P., 534
Kabler, P. W., 52, 527
Kafanova, N. D., 393
Kafer, E., 369
Kagan, I. G., 116
Kainski, J., 420
Kaiser, A. D., 42, 45
Kakimoto, D., 300
Kalinenko, V. O., 389
Kalinin, B. I., 402
Kalininskaya, T. A., 234
Kalle, K., 287
Kallio, R. E., 212
Kamen, M. D., 214
Kamien, N. E., see Niedergang-Kamien, E.
Kaminski, Z. C., 262
Kan, B., 510, 517
Kanazawa, A., 300
Kanazir, D., 313
Kandler, G., 18
Kandler, O., 18
Kaneko, T., 265
Kantarovich, R. A., 384
Kapikian, A. Z., 49, 66, 67, 68, 69
Kapitsa, O. S., 385
Kaplan, N. O., 152, 214
Kaplan, R. W., 374
Kapul, N. M., 384
Kapustiak, S. I., 401
Karel, M., 510
Karibian, D., 166, 186
Karnovsky, M. L., 166, 186, 264
Karpuzidi, K. S., 406
Kasatkina, G. V., 407
Kasatkina, I. L., 396
Kashiba, S., 5, 8
Kashiwada, K., 300
Kashkin, K. P., 386
Kassanis, B., 486
Katagiri, M., 167, 171, 190, 213

Kathan, R. H., 527
Kato, H., 266
Kato, K., 67
Kato, N., 267
Katsitadze, V. A., 396, 404
Katz, S., 50, 51, 55, 58, 59, 61, 63
Katznelson, H., 416
Kaudewitz, F., 349
Kaufman, M. J., 429
Kawagoe, K., 300
Kaye, S., 540, 541, 542, 543
Keeler, R. F., 234, 235, 255
Kefauver, M., 213
Kegeles, G., 82, 88, 91
Keilin, J., 224
Keitt, G. W., 372, 377, 416, 425, 428, 457, 458
Kellenberger, E., 5, 10, 17, 29, 30, 34, 35, 36, 40
Keller, J. R., 462
Kelliher, M. G., 509
Kelly, A. R., 544
Kelly, B., 51, 53, 54, 63, 64, 65
Kelner, A., 320, 508, 511, 512, 526, 527
Kellaway, C. H., 492
Kempe, L. L., 512, 514, 516, 517, 519
Kendall, E. J. C., 60, 61, 64
Kendrick, E. L., 377
Kennedy, R. E., 543
Kent, L. H., 163
Keppie, J., 79, 82, 83, 84, 87, 88, 89, 90, 91, 92, 95, 96, 97
Kerby, G. P., 502
Kershaw, W. E., 115
Kessel, J. F., 103
Kessler, E., 225
Keur, J. Y., 461
Key, A., 170, 183
Keyworth, W. G., 461
Khalil, M., 115
Khomiakov, A. M., 403
Khomik, S. R., 397
Khristov, A. C., 405
Khudairi, A. K., 238
Kian Joe Lie, see Lie, Kian Joe
Kienholz, J. R., 416
Kiese, M., 228
Kihara, H., 267
Kilborne, F. L., 136
Kilby, B. A., 170
Kilham, L., 66
Killisch, L., 18
Kimball, A. W., 373
Kimball, R. F., 314, 508
Kimura, S., 62
Kimura, S. J., 51, 61, 62, 63
Kinder, E., 108
King, C. G., 287
King, H. K., 88, 260
King, J. D., 535
King, J. H., 542, 543
King, M. E., 462
King, T. W., 153, 154, 187, 267
Kinsky, S. C., 220
Kirby, R. S., 416
Kirby, W. A., 426
Kirby-Smith, J. L., 112
Kirchner, C., 346
Kiritani, K., 318
Kirk, I., 319
Kirmse, T., 110
Kirn, J. F., 517
Kistiakowsky, G. B., 533
Kitahara, K., 265
Kitaoba, M., 66
Kitay, E., 266, 267
Kitayama, T., 66
Kitch, B. B., 149, 164
Kitching, J. A., 281
Kitiyakara, A., 351
Kivela, E., 535
Kizer, D. E., 251
Kjellen, L., 51, 52, 54, 55, 56, 57, 58, 61, 64
Klarenbeek, A., 541
Klarmann, E. G., 536, 539
Klausmeier, R. E., 165, 173, 226
Kleiger, B., 492
Klein, H. P., 184
Klein, R., 229
Klein, R. M., 479, 480
Kleinschmidt, A., 108
Klemmer, H. W., 418, 482, 483
Kliachko, N. S., 406
Klieneberger-Nobel, E., 17, 18
Kliewe, H., 533, 534
Kline, E. S., 355
Klinkowski, M., 419, 428
Kloetzel, M. C., 535
Klomparens, W., 418
Klotter, H. E., 282
Klungsöyr, L., 153
Kluyver, A. J., 208, 211, 212, 215, 216, 217, 223, 237
Knaysi, G., 2
Knivett, V. A., 155
Knizhnikov, V. A., 396
Knorr, L. C., 462
Knowlton, J. A., 509
Knox, J. M., 112
Knox, W. E., 535
Koch, G., 28, 29, 30, 31, 32, 33, 34, 35, 36
Koch, K. L., 444
Kodicek, E., 263
Koehler, B., 461
Kogut, M., 181
Koh, W. Y., 514, 521
Kohler, H., 419
Kohler, E., 449
Kohn, H., 511
Kojevnikov, P. V., 106
Kok, G., 54, 58, 63
Kokurichev, P. I., 398
Kolb, R. W., 541, 543
Kolchins'ka, I. D., 390
Kolesnik, Z. A., 395
Kolesnikov, G. F., 400
Kølmark, G., 319, 374
Komarovsky, B., 281
Kon, S. K., 253
Kondrat'eva, V. F., 386
Konikova, A. S., 402
Konishi, C., 237
Koniush, O. V., 406
Kono, M., 226
Konosh, O. V., 388
Konovaltchikoff-Mazoyer, M., 224
Konrich, F., 534
Kopech, G., 469
Kopytovskaia, L. P., 403
Korkes, S., 179
Kornberg, A., 179, 257
Kornberg, H. L., 155, 181, 182, 183, 248, 249, 258, 259
Korns, R. F., 106, 528
Korobkova, E. I., 405
Korosteleva, V. S., 403
Korotiaev, A. I., 393
Korzenovsky, M., 538
Koser, S. A., 251
Kosiakov, P. N., 403
Kotlán, S., 114
Kovalevskii, M. F., 403
Kozloff, L. M., 31, 34, 35, 39
Kozlovskaia, L. A., 402
Kozulitsina, T. I., 398
Krakow, G., 180
Krampitz, L. O., 179, 184, 190, 191
Krasil'nikov, N. A., 392, 393
Krasna, A. I., 224, 228, 253
Kratz, W., 285
Kratz, W. A., 237
Krause, G. A., 533, 534
Krauss, M., 328
Krauss, M. R., 326, 357
Kravitz, E., 535
Kraybill, H. R., 518
Krebs, H. A., 155, 178, 182, 183, 248
Krech, U., 528
Kridl, A. G., 533
Kriss, A. E., 394
Krog, A. J., 535
Kriukova, I. N., 400, 401
Krumholz, L. A., 288, 294
Krupka, L. R., 425
Krupp, I. M., 104
Kruse, K., 533
Kucera, S., 255
Kucherova, N. T., 398
Kuchinskas, E. J., 269
Kulagin, S. M., 399
Kulescha, Z., 472
Kumagai, T., 66, 67

Kummerow, F. A., 270
Kun, E., 105, 191
Kuna, A., 503
Kunkel, H. G., 335
Kunkel, L. O., 442
Kuntz, J. E., 430
Kuntz, R. E., 115, 116, 117
Kupferberg, L. L., 165
Kurahashi, K., 179, 355
Kurek, L. I., 353, 369
Kuritsina, N. M., 384
Kuroya, M., 66, 67, 533
Kurtzman, R., 486
Kustoff, T. Y., 267
Kutsky, R., 486
Kuznetsova, N. I., 403
Kuznetsova, V. A., 395
Kvesitadze, I. F., 402
Kylin, A., 288
Kylin, H., 288

L

Lacey, R. J., 117
Lack, C. H., 496, 499, 500, 504
Lackey, C. F., 442, 451
Lafferty, J. W., 113
Lagermalm, G., 52, 56, 57 58
Laing, A. B. G., 111
Laki, K., 533
Lamanna, C., 531
Lambert, J., 536
Lambina, V. A., 394
Lampen, J. O., 148
Landman, O., 15
Landman, O. E., 6, 10, 15
Lang, D. A., 510
Lankford, C. E., 266, 267
Lannon, T. J., 167
Lapinleimu, K., 55, 60, 61, 64
la Rivière, J. W. M., see Rivière, J. W. M. la
Lark, K. G., 324
Lascelles, J., 210
Laskowski, W., 375
Lasserve, C. T., see Tournier-Lasserve, C.
Latarjet, R., 320, 334
Latyshev, N. I., 106
Laurent, T., 164
LaVeck, G. D., 504
Laverde, A. G., see Garcia-Laverde, A.
Lawless, D. K., 105
Lawrence, C. A., 535
Lawrence, J. J., 115
Lawton, E. J., 510
Lawton, W. D., 95
Layne, E. C., 236
Lazarev, N. V., 395
Lea, D. E., 510
Leat, W. M. F., 263
Leben, C., 416, 425, 428, 431
Lebovitz, J. L., 497
Lederberg, E. M., 336, 350, 355
Lederberg, J., 5, 10, 11, 17, 18, 19, 20, 34, 36, 43, 311, 324, 335, 336, 340, 341, 342, 344, 347, 350, 355, 356, 370, 371
Ledingham, G. A., 486, 487
Lee, H. H., 32, 33
Lee, S. B., 231
Lees, H., 229
Lee-Whiting, B., 369
Lefèvre, M., 302
Le Gac, P., 115
Lehman, J. J., 527
Leidy, G., 326, 334, 357
Leloir, L. F., 161, 264
Lelong, M., 50, 63
Lemaigre, C., 115
Le Minor, L., 340
Le Minor, S., 340
Lemon, H. M., 539
Lenhoff, H. M., 214
Lennette, E. H., 51, 55, 58, 59, 63
Lennox, E. S., 45
Lepine, P., 50, 57, 63
Lepow, I. H., 111
Lerman, L. S., 330, 333
Lerner, P., 354
LeRoux, P. M., 377
Lester, G., 353
Lester, W., Jr., 539
Leuchtenberger, C., 54, 56, 57
Leupold, U., 366, 367, 372
Le-Van Phung, 114
Levashev, V. S., 388
Levi, L., 494
Levin, D. H., 269
Levine, M., 44, 337
Levinson, H. S., 270
Levinthal, C., 39, 41, 42, 43, 322
Levkovich, E. N., 400
Levy, E., 493
Levy, H. B., 56
Lewin, J. C., 286, 298
Lewin, R. A., 296, 299, 300, 302
Lewis, G. B., 352
Lewis, G. J., 288
Lewis, I. M., 266
Lewis, J. C., 267
Lewis, L., 301
Lewis, L. J., 528
Lewis, R., 103
Li, K., 357
Li, T. K., 15
Lialikova, N. N., 394
Liberman, M., 56
Licciardello, J. J., 518, 521
Lichstein, H. C., 267
Lichtenstein, J., 256
Lida, K., 221
Lidwell, O. M., 539
Lie, Kian Joe, 114
Lieb, F., 533
Lieb, M., 337
Liebermeister, K., 5, 10, 17
Lieske, R., 212
Limasset, P., 485
Lincicome, D. R., 105
Lindeberg, G., 164
Lindegren, C. C., 366, 368, 371, 372, 373
Lindegren, G., 366, 368, 372, 373
Lindenmeyer, J., 534
Lineweaver, H., 520
Link, K. P., 482
Linstead, R. P., 168, 170
Lipkin, M. E., 404
Lipmann, F., 179, 181
Lippelt, H., 61, 64
Lister, C. A., 461
Litman, R. M., 316
Little, H. N., 228, 231
Little, R. M., 495
Liu, C., 70
Liu, G. B., 15
Livadas, G. A., 112
Lloyd, P. J., 421
Lloyd, R. P., 249
Locke, S. B., 478
Lockhart, E. E., 518, 520, 521
Lockhead, A. G., 203, 265
Lockwood, J. L., 422, 425, 427
Lockwood, S., 289, 290
Lodge, A. L., 497
Lodge, R. M., 536
Loebeck, M. E., 272
Loeding, J. W., 509
Loefer, J. B., 296
Lofgren, R., 108
Loginova, L. G., 386
LoGrippo, G. A., 544
Logsdon, C. E., 424
Lohss, F., 28, 29, 30
Lokhov, M. G., 398
Lomteva, M. N., 391
Long, M. V., 179, 183
Loosli, C. G., 51, 54, 55, 58, 60, 65, 539
Lopes, L. A., see Assis-Lopes, L.
Lorenz, F. W., 542
Lorenz, L. L., 497
Losada, M., 370
Louttit, C. M., 103
Love, R., 58
Lovelock, J. E., 539
Low, B. W., 57
Lowenberg, J. R., 164
Lozinov, A. B., 389
Lozinsky, E., 543
Lucas, C. E., 279, 302
Lucas, G. B., 426
Lugenbuhl, R. J., 66

Luippold, H. E., 313
Luisada-Oppen, A. V., 251
Lu Ju-Shan, 391
Lukton, A., 262
Lumb, G. D., 536
Lund, H., 113
Lund, J. W. G., 281, 294
Lunde, M. N., 109
Lunseth, J., 110
Luria, S. E., 27, 40, 338, 346, 358, 442
Lute, M., 35
Lutsenko, T. A., 402
Lwoff, A., 27, 44, 291, 295, 296, 297, 299, 338
Lynen, F., 190, 191
Lyons, C., 495

M

Maaloe, O., 324
Maas, W. K., 261
McAnelly, J. K., 266
McAuliffe, C., 225
McBee, R. H., 160
McBryde, C. N., 142
Maccacaro, G. A., 340
McCallan, S. E. A., 270
McCallum, A. H., 238
MacCallum, P., 492
McCarthy, J. F., 287
McCarthy, M. A., 53
MacClement, W. D., 462
McClure, T. T., 417
McCombie, A. M., 292
MacConnell, J. T., 238
McConnell, W. B., 207
McCullough, N. M., 51, 60, 61, 62
McCune, R. M., Jr., 502
MacDirmid, A., 81, 97
MacDonald, D. L., 168
McDonald, I. J., 250
McDonald, J. C., 50, 58, 60, 61, 63, 64
McDonald, J. R., 452
McElroy, W. D., 204, 211, 219, 220, 227
McEvoy, D., 254
MacFarlane, J. O., 532
Macfarlane, W. V., 117
McGee, J., 147, 190
McGhee, W. J., 82, 88, 90
McGrew, J. R., 462
Mach, F., 421
Machlis, L., 265
Macias, F. M., 271
McIntosh, E. N., 187
Mackaness, G. B., 86
Mackay, I., 539
Mackay, J. H. E., 433, 434
Mackie, T. T., 105, 117
Mackinney, G., 262
McKinney, H. H., 449, 461
Mackinnon, J. P., 462
McLaughlin, C. B., 532
McLaughlin, J. J. A., 284, 285, 286, 289, 292, 293, 294, 297, 298
McLean, I. W., Jr., 52
McLean, R. A., 518
MacLennan, A. P., 166
Macleod, C. M., 77
MacLeod, C. M., 326, 328, 357
McLeod, D. L., 67
MacLeod, R. A., 251, 255
Macmillan, A., 204, 205, 206, 207, 221
McMullen, D. B., 117
McNall, E. G., 224, 250
Macpherson, I. A., 98
McQuillen, K., 1, 3, 5, 6, 9, 10, 14, 16, 258
MacRae, I. C., 213
MacVicar, R., 207
McWhorter, F. P., 461
Madoff, S., 18
Madsen, N. B., 181, 182, 183, 249
Maegraith, B., 104, 111
Magasanik, B., 166, 186, 257, 260, 261, 268, 356
Magee, W. E., 233, 234
Magness, J. R., 453
Mahler, H. R., 5, 6, 8, 11, 34, 152, 347
Mahn, G. R., 509
Maier, C. R., 429, 435
Maiskii, I. N., 404
Makela, P., 55
Maksimova, I. V., 390
Makstenieks, O., 60
Malakatis, G. M., 115
Malcolmson, J. F., 423, 424
Malis, G. I., 400
Mallette, M. F., 531
Mallman, W L., 535
Mamatsashvili, E. G., 385
Mandel, H. G., 258, 269
Mandels, M., 161, 260
Mankau, S. K., 118
Mann, S. O., 163, 166
Manns, M. M., 462
Manns, T. F., 462
Manolov, D G., 397
Mantel, N., 105
Maplestone, P. A., 112, 113
Marcovich, H., 320
Markham, R., 42, 258
Markitantova, A. E., 402
Markov, K. I., 255
Marks, J., 493
Marlatt, R. B., 420
Marmur, J., 326, 327
Marrian, D. H., 247
Marshall, C. G., 535
Marth, P. C., 453
Mathews, I., 452
Martin, A. V., 333
Martin, C., 486
Martin, J. K., 254
Martin, S. P., 502
Martin, W. R., 192, 259, 260
Martinez, D., 301
Martinez, M., 227
Martyniuk, I. V., 386
Mase, Y., 263
Mashkov, A. V., 398
Masilungan, V. A., 117
Maslenikova, L. K., 406
Mastrota, F. M., 49, 66, 67, 68, 69
Matheson, B. H., 494
Mathews, J., 541
Mathews, J. N., 426
Mathieson, M. J., 370, 372
Matsubayashi, R., 217
Matsuzawa, M., 66
Matthews, R. E. F., 257
Matudaira, T., 286
Maurice, P., 536
Maurin, J., 57
Mauris, C., 51, 58, 59, 63
Mayall, R., 114
Mayer, A., 443
Mayhew, R. L., 112, 113
Maylor, H. B., 250
Mazé, R., 345
Mazia, D., 321
Mazilkin, I. A., 394
Mazoyer, M. K., see Konovaltchikoff-Mazoyer, M.
Meadow, P., 5, 9, 10, 271
Medina, A., 223, 225, 226
Mehler, A. H., 179, 184
Meier, P., 528
Meiklejohn, G., 70
Meira, J. A., 115
Meisel', M. N., 391
Meister, P. D., 263
Mekhtiev, S. I., 394
Melechen, N., 39, 44
Meleney, H. E., 117
Melkumova, T. A., 385
Mel'nik, E. G., 397
Melton, M. L., 109
Melvin, D. M., 103
Mendelevich, M. M., 403
Mendenhall, E. E., 534
Menkin, V., 496, 502
Mercer, R., 113
Mergenhagen, S. E., 250
Merriam, H. E., 138
Meselson, M., 324
Metcalfe, G., 237
Meyer, E., 81, 96
Meyer, H., 108
Meyer, K. F., 79, 81, 95, 96
Miagkaia, I. P., 407
Michaelis, M., 167
Mickelsen, O., 542
Migliacco, A., 253
Mikhailov, I. F., 406
Mikhailova, I. F., 402
Mikhalenko, Z. V., 403
Mikulin, M. A., 395
Milbrath, J. A., 457, 459
Miles, A. A., 77, 78, 82, 85, 86, 94, 98, 501, 502

Miles, E. M., 85, 86, 502
Miles, P. G., 374
Militzer, W. E., 16
Millar, R. L., 428, 434
Miller, B. F., 539
Miller, C. O., 469, 475, 476
Miller, C. S., 257
Miller, D. A., 251
Miller, H. N., 430
Miller, J. W., 106
Miller, L. P., 270
Miller, M. J., 103
Miller, P. M., 416
Miller, W. C., Jr., 512, 513
Millikan, D. F., 462
Mills, G. T., 161, 162
Mills, W. D., 416, 456, 457
Milner, H., 303
Minami, K., 270
Mineeva, L. V., 388
Minghetti, A., 253
Minner, J. R., 51, 56
Minning, W., 116
Minoda, R., 62, 65
Minor, F. W., 156, 157, 158
Minuse, E., 67
Mirzabekian, R. O., 418
Mishustin, E. N., 393
Mitchell, H. K., 252, 365, 366, 368, 369, 370, 373
Mitchell, J. W., 415, 419, 420, 431
Mitchell, M. B., 366, 368, 369, 370, 373
Mitchell, P., 1, 4, 5, 7, 8, 11, 12, 14, 16, 193, 247
Mitchell, R. L., 288
Mitina, T. V., 407
Mitsui, Y., 62, 63, 65
Miyazaki, I., 114
Mogabgab, W. J., 49, 70, 71
Mohler, J. R., 131
Monlux, W. S., 109
Monod, J., 154, 165, 173, 193, 247, 259, 268
Monsour, V., 236
Montgomery, M., 531
Montgremier, H. de A., see Augier de Montgremier, H.
Montoya, C., 112
Moody, M. D., 325
Moore, D. H., 52, 56, 56, 58
Moore, J. D., 455, 457, 458
Moore, V. A., 135
Mordashev, S. R., 391
Morehouse, C. T., 514, 521
Morel, G., 473, 474, 484, 485, 486
Moreno, L., 110
Morgan, B. H., 513, 514, 516
Morgan, C., 52, 56, 57, 58
Morgan, M. E., 250
Mori, T(akako), 231
Mori, T(akeshi), 217
Morioka, T., 5, 8
Morita, T., 300
Moroz, A. F., 386
Morris, J. A., 49, 69
Morris, W. T., 416, 418
Morrison, J. F., 188
Morse, H., 60
Morse, M. L., 335, 336, 350
Mortensen, J. D., 532
Morton, A. G., 205, 206, 207, 221
Morton, H. E., 530, 531
Morton, T. C., 103
Moser, H., 317
Moses, V., 179
Moss, V. D., 430
Most, H., 105, 106
Mouromseff, G., 539
Moyle, J., 1, 4, 5, 7, 8, 11, 12, 14, 16
Mozen, M. M., 232
Mudd, S., 18, 271, 530
Mudd, S. H., 258
Muhrer, M. E., 226
Muir, E., 476, 477, 486
Muir, R. D., 263
Muir, W. H., 472, 483, 484
Mukai, F., 373
Mukherjee, M. K., 237
Mulder, D., 459
Muller, K. O., 433, 434
Mullet, R. P., 426
Munekata, K., 67
Munkres, K., 369
Murano, H., 434
Murneek, A. E., 416
Murphy, J. F., 173, 174
Murphy, P. A., 445
Murray, E. G. D., 443
Murray, E. S., 63
Murray, H. C., 263
Murray, M. R., 469
Murray, N. A., 114
Murty, G. G. K., 270
Mutsaars, W., 4, 7, 10
Myatt, A. V., 111
Myers, J., 206, 237, 285
Myers, R., 49, 69

N

Nabokov, I. S., 386
Nagao, M., 114
Nagy, R., 539
Najjar, V. A., 212, 215, 216
Nakagawa, T., 339
Nakao, T., 66
Nakayama, T., 262
Nakhimson, L. I., 405
Napier, E. J., 432
Napier, J. A., 528
Napier, L. E., 113
Nashke, M. D., 180
Nason, A., 203-46; 153, 205, 210, 215, 216, 218, 219, 221, 222, 223, 225, 226, 227, 232, 234, 236, 270
Nathan, H. A., 289, 290
Nathan, H. C., 269
Natti, J. J., 422, 427, 435
Nauck, E. G., 103
Nayder, F., 271
Neas, M. O., 426
Nečas, O., 21
Nechaev, V. V., 406
Neeb, O., 287, 288
Neff, B. J., 55
Neidhardt, F. C., 260, 261, 356
Neilands, J. B., 263, 265
Neilsen, N., 267
Neish, A. C., 149, 164
Neisser, M., 533
Nelson, E. W., 288
Nelson, N. J., 320, 352
Nelson, N. M., 160
Nelson, P. R., 295
Nelson, R., 423, 429, 443
Nelson, R. R., 377
Nelson, T. C., 342
Neva, F. A., 51
Nevill, A., 206, 217
Newman, L. B., 541, 542
Newmeyer, D., 351
Newton, B. A., 535
Newton, W. L., 116
Nichol, C. A., 252
Nichols, R. L., 113
Nicolas, A , 51, 62, 63
Nicholas, D. J. D., 210, 214, 218, 219, 223, 225, 226,
Nickell, L. G., 483, 485
Nickerson, J. T. R., 518, 519, 520, 521
Niedergang-Kamien, E., 476
Nienow, I., 431
Niles, W. B., 142
Nisbet, M., 302
Nishigaki, S., 237
Nishihara, H., 535
Nishikawa, F., 66
Nisman, B., 5, 15, 16
Nitowsky, R. T., 64
Nitsch, C., 477
Nitsch, J. P., 477
Niven, C. F. Jr., 507-24; 267, 271, 508, 511, 513, 514, 518, 521
Niven, J. S. F., 501
Niwa, M., 209
Nobecourt, P., 475
Nobel, E. K., see Kliene-berger-Nobel, E.
Noblesse, M., 164
Nomura, M., 8, 190, 191
Nord, F. F., 161
Nordan, H. C., 514, 515
Norden, H. C., 513
Nordgren, G., 538
Nordli, E., 286
Norris, E. R., 168
Norris, J. R., 255
North, L. L., 530, 531
Noskova, E. G., 387
Novelli, G. D., 179, 253
Novick, A., 310, 317

Nungester, W. J., 86
Nutman, P. S., 177
Nutting, L. A., 149
Nygard, J. C., 509

O

Oberholzer, P. C. J., 452
Oberle, E. M., 510, 512, 513
O'Brien, R. T., 270
Ocfemia, G. O., 462
Ochi, M., 66
Ochoa, S., 146, 153, 179, 180, 236
Odom, V., 105
Offutt, A. C., 103
Ogata, S., 62, 65
Ogawa, J. M., 418
Ogg, J. E., 311, 346
Oglesby, G., 266
Ogston, A. G., 185
Ogur, M., 366, 373
Ohashi, K., 66
O'Hea, A. J., 77
Ohmachi, K., 221
Ohmura, E., 190, 260
Ohmura, H., 223
Oikawa, T., 93
O'Kane, D. J., 145, 179
Okaniwa, A., 67
Oker-Blom, N., 55, 60, 61, 64
Okumura, F. S., 475
Okuno, Y., 66
Olafson, P., 109
Olenberg, G., 502
Olive, L. S., 368, 372, 376
Oliver-Gonzalez, J., 116
Olivier, L., 117
Olivo, J. P., 514
Olson, E. J., 163
Olson, E. O., 452
Olson, J. A., 181, 183
Onofrey, E., 255
Onvlee, P. C., 542
Oparin, A. I., 4, 7, 10, 14, 390
Oppen, A. V. L., see Luisada-Oppen, A. V.
Orgel, L. E., 350
Orkin, B. A., 170
Orla-Jensen, A. D., 267
Orla-Jensen, S., 267
Ormsby, H. L., 50, 60, 61, 62, 63
Ortenzio, L. F., 541
Oshima, H., 66
Osipova, P. V., 396
Osterud, K. L., 296
Ottey, L., 167
Otto, G. F., 105
Overhulse, P. R., 544
Oxford, A. E., 163, 166
Ozeki, H., 335, 349

P

Packalen, T., 500
Paden, W. R., 461
Paffenbarger, R. S., 51, 52, 61, 64, 65
Pahl, H. B., 332
Paine, J., 461
Painter, H. A., 230
Painter, R. B., 323
Palleroni, N. J., 151, 190
Palmiter, D. H., 458
Palmstierna, H., 164, 165
Panijel, J., 5, 8
Panisset, M., 262
Pankova, S. S., 402
Panos, C., 262
Papazian, H. P., 374
Papirmeister, B., 116
Pappas, I., 286
Pappenheimer, A. M., Jr., 77
Pardee, A. B., 1, 20, 194, 259, 261, 316, 347
Park, J. T., 20, 34, 163
Parker, C. A., 238
Parker, E., 52, 56, 57
Parker, K. G., 452, 453, 454, 456, 458
Parker, R. C., 469, 487
Parks, L. W., 252, 369
Parodi, S E., 114
Parrish, G., 103, 514, 515
Parrish, M., 103
Parrott, R. H., 50, 51, 52, 53, 54, 55, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69
Partridge, C. W. H., 320, 352
Pasteur, L., 91
Pastukhova, G. M., 399
Patel, M. D., 167, 170, 171, 183
Pateman, J. A., 351, 369, 370
Patterson, M., 52
Pattison, D. B., 170
Patty, F. A., 543
Paulus, A., 419
Payne, A. M. M., 49, 50, 51
Payne, F. M., 103, 106
Payne, J. I., 271
Pazur, J. H., 165, 166
Pchelkina, A. A., 399
Pearsall, W. H., 281, 283
Pease, P., 18
Pedigo, P. R., 366
Peck, H. D., Jr., 188, 189, 230, 231, 233
Peel, E., 115
Peel, J. L., 145-202
Pelet, F., 483
Pelevina, M. V., 386
Pelon, W., 49, 70, 71
Pengra, R. M., 232
Penner, L. R., 117
Pepper, R. E., 514, 521
Pereira, H. G., 51, 53, 54, 55, 61, 63, 64, 65
Pereira, J. N., 250
Peremitina, L. D., 385
Peres, C., 105
Perkins, B. L., 424
Perkins, D. D., 371, 372
Perkins, J. J., 542
Perlingiero, J., 113
Pervachenko, S. V., 404
Pesigan, T. P., 117
Petersen, D., 417
Petersen, R. A., 289, 290
Peterson, D. H., 263
Peterson, D. R., 286
Peterson, E. A., 428
Peterson, H. I., 177
Peterson, R., 267
Peterson, W. H., 430
Petit, J. F., 264
Petkus, E. J., 509
Petrosian, A. P., 390
Pfander, W. N., 226
Phelps, W. R., 430
Phillips, B. P., 103, 104, 105
Phillips, C. R., 525-50; 534, 537, 538, 540, 541, 542, 543
Phillips, D. M., 333
Phillips, I. A., 49, 70, 71
Phillips, J. H., 335
Phillips, L., 105
Phillips, M. M., 366
Phung, Le-Van, see Le-Van Phung
Pichinoty, F., 224
Piekarski, G., 109
Pierce, C. H., 81, 93
Pierce, W. E., 49, 58, 70, 71
Pifano, F., 107
Pillai, V. K., 287
Pille, E. R., 406
Pillemer, L., 111, 492
Pimental, J., 115
Pinchot, G. B., 152
Pinnock, P. R., 57
Pinsky, A., 167
Pintner, I. J., 279, 283, 284, 285, 286, 297, 298, 300, 302
Piriatinski, L. B., 401
Pirson, A., 287
Pittenger, T. H., 373
Pittman, D. D., 366, 373
Pitts, T. D., 115
Pivnick, H., 247
Pizzi, T., 108, 110
Plakidas, A. G., 462
Plaut, W., 321
Plescia, O., 335
Pletsityi, D. F., 404, 407
Podoski, E. P., 181
Poetschke, G., 18
Pokrovskaia, N. V., 390
Pollack, M. R., 217
Pollard, J. K., 475
Pollitzer, R., 79, 95
Pomales, A. B., see

Brenes-Pomales, A.
Ponte, E. del, 106
Pontecorvo, G., 365, 367, 368, 369
Pope, S., 254
Popova, L. A., 392
Poppensiek, G. C., 541
Porat, T. B., see Ben-Porat, T.
Portelance, V., 262
Porter, K. R., 108
Porterfield, V. T., 239
Portmann, A., 267
Posnette, A. F., 457
Postgate, J., 214
Potter, R. L., 272
Poulos, P. L., 416
Povalishina, T. P., 106
Powell, H. M., 532
Powell, J. F., 163
Powers, H. R., Jr., 428
Pradatsundarasar, A., 114
Pramer, D., 432, 433, 434
Pratt, D. M., 295
Prescott, D. M., 351
Prescott, G. C., 417
Prestidge, L. S., 20, 347
Preston, W. H., 431
Prezyna, A., 110
Price, D. L., 115
Price, S. A., 493
Price, W. C., 442, 448, 449
Price, W. H., 49, 70
Prickett, P. S., 542
Prieto, A. P., 147
Prigal, S. J., 503
Prijyanonda, B., 114
Primosigh, J., 20, 28, 32, 33, 34
Pringsheim, E. G., 206
Prior, J. A., 109
Pritchard, R. H., 368, 369, 370
Proctor, B. E., 509, 510, 512, 513, 517, 518, 519, 520, 521, 522
Proctor, V. W., 206, 303
Prommas, C., 114
Proom, H., 325
Protsenko, A. E., 384
Provasoli, L., 279-308; 279, 283, 284, 285, 286, 288, 289, 295, 296, 297, 298, 299, 300, 301, 302, 310
Prusoff, W. H., 258
Pshenichnov, A. V., 398, 399
Ptokhov, M. P., 400
Puchett, N., 271
Puchkov, N. V., 401
Puck, T. T., 29, 30, 32, 33, 35, 36, 539
Pulvertaft, R. J. V., 536
Purko, M., 187

Q

Quadling, C., 310, 351
Quakenbush, F. W., 263
Quastel, J. H., 177
Quince, P., 146
Quinn, D. H., 534

R

Rabil, P. J., 542
Rabin, R., 258
Rabinowitz, J. C., 267
Rabourn, W. J., 263
Racker, E., 148, 158
Radionova, L. N., 406
Raffel, S., 77
Rahn, O., 536
Raichel, B., 2
Rakestraw, J. A., 233, 270
Rakhno, P. K., 237, 384
Raleigh, W. P., 453
Rammelkamp, C. H., Jr., 497
Ramsey, H. H., 266, 267
Randle, C. J. M., 166
Ranftl, J. W., 509
Rangaswami, G., 428
Rankin, H. W., 447
Rao, C. B., 281
Rao, V., 114
Raper, J. R., 374, 375
Rappoport, D. A., 148
Rappaport, J., 484
Rashba, E. I., 390
Rausch, R., 117
Ravel, J. M., 266
Ravenholt, R. J., 504
Ravin, A. W., 309-64; 326, 357
Rawlins, T. E., 431, 452, 486
Rawson, D. S., 282
Raymond, W. F., 539
Read, T. R., 104, 106
Reardon, L. V., 103, 104, 105
Reddish, G. F., 529, 530, 532, 537
Reed, J. M., 514, 516
Reed, L., 542
Reed, L. J., 265
Rees, C. W., 103, 104, 105
Reese, E. T., 161, 164, 260
Reeves, E. L., 457, 458
Reich, K., 292
Reichel, L., 263
Reichelderfer, T., 49, 66, 67, 68, 69
Reid, E. E., 536
Reid, J. J., 177
Reiman, J., 503
Reiner, B., 316
Reinert, J., 470, 478
Reinertson, J. W., 104
Reio, L., 179
Rendtorff, R. C., 104
Renjifo, S., 107
Repaske, R., 5, 7, 153, 194
Resag, K., 228
Reyniers, J. A., 103
Rhea, W. G., Jr., 269
Rheins, M. S., 504
Rhodes, A., 432
Rhodes, A. J., 67
Rhodes, G. N., 426
Rhuland, L. E., 271
Rice, T. R., 288
Rich, A., 41
Rich, K., 316
Rich, S., 427
Richards, B. L., 458
Richards, E. L., 266
Richards, M. G., 462
Richardson, E. M., 536
Richter, A., 340, 342, 358
Richter, G. H., 533
Rickenberg, H. V., 5, 8 194, 247, 259, 353
Richert, D. A., 222, 228
Riddle, K. B., 542
Riddle, R. W., 60, 61, 64
Rideal, S., 529
Rieman, G. H., 487
Rier, J. P., 473
Riker, A. J., 469-90; 418, 430, 470, 472, 476, 477, 478, 479, 480, 481, 482, 483, 484, 486
Rilling, H., 262
Rita, G., 338
Ritchie, L. R., 117
Ritchie, L. S., 103, 117
Rittenberg, D., 224, 228
Riviere, J. W. M. la, 190, 191, 192
Robbins, K. C., 492
Robbins, W. C., 335
Robbins, W. J., 297, 301, 471, 486
Roberts, C., 366, 367, 370
Roberts, E. R., 233, 234, 270
Roberts, J. L., 542
Roberts, M. H., 536
Roberts, R. B., 179, 184, 205
Robertson, O. H., 539
Robichon-Szulmajster, H. de, 355
Robinow, C. F., 1, 311, 370
Robinson, D. B., 423
Robinson, R. S., 429, 430, 432, 434
Robinson, T. A., 104, 106
Robles, H., 112
Rochat, G., 533
Rodan, K. S., 60, 61, 64
Rode, L. J., 266
Roden, A. T., 54, 61, 65
Rodger, W. A., 509
Rodriguez, E., 257, 289, 290
Roe, A. S., 326, 357
Rogers, D., 267
Rogers, D. E., 495, 496, 502

Rogers, H. J., 165, 500
Rogoff, M. H., 174, 177
Rohde, W., 283, 292, 293, 294
Roizman, B., 49, 54, 55, 56, 69
Román, C., 110
Roman, D. P., 270
Roman, H., 366, 367, 368
Romans, I. B., 534
Romig, W. R., 314
Romine, M., 542
Roof, B. S., 167
Roper, J. A., 369, 370
Rose, D., 267
Rose, H. M., 52, 56, 57, 58
Rose, I. A., 153, 236
Roseman, C., 109
Roseman, S., 166
Rosen, L., 56
Rosenblum, C., 253
Rosenblum, E. D., 231
Rosenthal, S. M., 268
Ross, G. I. M., 299, 300
Ross, J. D., 98
Ross, O. A., 111
Rostorfer, H. H., 228
Rothberg, S., 167, 213
Rothrock, J. W., 425
Rotov, I. V., 405
Rountree, P. M., 504
Roussos, G. G., 226
Rowatt, E., 265
Rowe, W. P., 49-76; 50, 51, 52, 53, 54, 55, 57, 58, 59, 60, 61, 62, 63, 64, 65
Rowell, J. B., 377
Rowlands, D. A., 247
Rowley, D., 3, 341, 342, 358
Roy, A. B., 237
Ruban, E. L., 229, 230, 233, 390
Rubio Huertos, M., 18
Ruinen, J., 237
Rupert, C. S., 320
Rush, D., 166, 186
Russell, P. F., 106, 107, 111, 112
Russi, M., 338
Ryan, F. J., 317, 318
Ryan, R. W., 51, 63, 64, 65
Rybakova, S. G., 395
Ryden, F. W., 105
Rydon, H. N., 81
Rylkin, S. S., 390
Ryther, J. H., 292, 293, 296
Ryzhkov, V. L., 391

S

Sabet, K. A., 428
Sabin, A. B., 109
Sacks, L. E., 212, 213, 216
Sadana, J. C., 211
Sadun, E. H., 104
Saev, G. K., 255
Sagae, K., 66
Sagers, R. D., 189
Sagik, B., 30
Saiki, E., 543
St. John, R., 366, 373
St. Lawrence, P., 367, 368, 369, 370
Saito, H., 366
Saito, Y., 190
Sakaguchi, K., 374
Sakhnovskaia, G. K., 393
Sakurogi, T., 270
Salk, J. E., 528
Sall, T., 271
Salle, A. J., 530, 533, 538
Salton, M. R. J., 1, 2, 3, 4, 5, 6, 8, 10, 14, 84, 163, 535
Saluste, E., 179
Sanders, E. P., 104
Sanders, F., 252
Sanders, M., 285, 289, 290
Sanders, R. T., 526
Sands, S. M., 366
Sansome, E., 368, 369
Santer, M., 146
Santer, U. V., 353
Saper, E., 405
Sapero, J. J., 103, 105
Sappenfield, R. W., 103, 106
Sargeant, K., 88, 90, 92
Sarachek, A., 271
Saris, N. E., 225
Sarma, P. S., 266
Sasahara, J., 66, 67
Saslaw, S., 109
Sato, R., 208, 209, 211, 215, 223
Saunders, G. W., 302
Savage, R. E., 49, 69
Savan, M., 541
Sawitz, W., 105
Sayad, W. Y., 114
Sayre, C. B., 422
Saz, A. K., 227
Saz, H. J., 181, 183, 184, 249
Scawin, J. H., 219
Schachman, H. K., 1
Schaechter, M., 259
Schaedler, R. W., 503
Schaefer, W. B., 81, 93
Schaeffer, F. L., 529
Schaeffer, P., 326, 327, 330, 331, 357, 358
Shaffer, T. E., 504
Schaffner, C. P., 263
Schar, M., 95
Schatten, W. E., 58
Schatz, A., 272, 288, 310
Scheider, M., 534
Schellenberg, H., 17, 18
Schenone, H., 112
Scher, S., 285, 289, 290
Scherp, H. W., 250
Schiller, E. L., 117
Schlegel, D. E., 431
Schleich, F., 108
Schmidt, C. F., 516
Schneider, C., 228
Schneider, M. C., 180
Schneiter, R., 541, 543
Schoenleber, A. W., 106
Schramm, M., 148, 155, 156, 157, 158, 159, 160
Schricker, J. A., 542
Schrodter, H., 419
Schubert, W. J., 161
Schuhardt, V. T., 266
Schul'man, E. S., 114
Schulman, H. M., 5, 10, 19, 347
Schultz, E. S., 443
Schultz, H. W., 513
Schulz, I., 181
Schwab, J. H., 94, 95
Schwartz, A. M., 156, 157
Schwartz, G., 534
Schweigert, B. S., 271, 518
Schwenk, E., 263
Schwerdt, C. E., 529
Schwetz, J., 115
Scott, C. E., 416, 444
Scott, D. B. M., 324
Scott, D. H., 462
Scott, G. D., 237, 291
Scott, R. W., 482
Seal, J. R., 51, 58, 59
Seaman, G. R., 180, 225, 252
Sèchaud, J., 40
Segal, N., 82, 86, 92, 93
Segal, S., 166
Segretain, G., 485
Seidelin, R., 103
Seidler, A. J., 271
Seki, Y., 5, 8
Seliber, L. G., 389
Selimov, M. A., 403
Selzer, L., 531
Semcheva, N. S., 389
Semenov, B. F., 400
Semenova, E. V., 403
Sen, K., 114
Senez, J. C., 224
Sergeant, T. P., 267
Sergeeva, R. V., 213
Sermonti, G., 346, 374
Sermonti, I. S., see Spada-Sermonti, I.
Serres, F. de, 367, 369
Serres, F. J. de, 367, 369
Setlow, J. K., 300
Sexton, R. J., 543
Sforza, L. L. C., see Cavalli-Sforza, L. L.
Shablovskaia, E. A., 393
Shaefer, W. B., 270
Shaffer, J. G., 105
Shamaeva, E. M., 402
Shan, L. J., see Lu Ju-Shan
Shantz, E. M., 469, 474, 475
Shapiro, H. S., 5, 10, 19, 347
Shapiro, J., 302
Shapiro, R. L., 534

Sharp, J. T., 18
Sharpe, H. S., 374
Shaughnessy, H. J., 535
Shaw, L., 426
Sheehan, H. L., 93
Sheffner, A. I., 266
Sheinker, A. P., 387
Shelokov, A., 68
Shemanova, G. F., 391
Shepard, P., 417
Sherman, H. J., 104, 105, 106
Sherman, J. M., 267
Shertsl, I., 402
Sheveleva, O. N., 387
Shih, B. C., 105
Shilo, (M)iriam, 292
Shilo, (M)oshe, 190, 191, 192, 193, 194, 259, 292
Shimazono, H., 190
Shimwell, J. H., 156
Shiraki, M., 227
Shiratori, T., 66, 67
Shirk, H. G., 156, 157, 158
Shive, W., 268
Shmonova, N. I., 395
Shneerson, A. N., 401
Shockman, G. D., 267
Sholl, L. B., 141
Shonnard, C. P., 544
Shooter, K. V., 333
Shorin, V. A., 392, 402
Short, R. B., 116
Shternov, V. A., 536
Shu, P., 164
Shuey, E. W., 165, 166
Shuey, H. E., 51, 58, 59, 63
Shug, A. L., 230, 231, 347
Shugaeva, N. V., 389
Shult, E. E., 368, 371
Shvaiko, V. A., 397
Sibray, W. S., 419
Sienkiewicz, H. S., 105
Sih, C. J., 160
Siim, J. C., 109
Sijpesteijn, A. K., 160
Silber, G., 420
Sil'chenko, V. S., 398
Silver, W. S., 222, 227
Silverman, W. B., 427
Simon, W. R., 494
Simms, B. T., 128
Simms, E. S., 257
Simmonds, S., 251
Simon, T., 110
Simpson, F. J., 149, 164
Simpson, See Hope Simpson, R. E.
Simpson, J. R., 229
Sims, P., 170
Singer, T. P., 531
Singh, R. N., 281, 291
Singh, S., 114
Sirnik, I. O., 396
Sironval, C., 236
Sison, A., 114
Sison, B. C., 161
Sistrom, W. R., 14, 168, 356
Skaar, P. D., 340, 344, 349, 350
Skeehan, R. A., Jr., 542, 543
Skeggs, H. R., 257
Skerman, V. B. D., 213
Škoda, J., 258
Skoog, F., 283, 285, 300, 469, 472, 475, 476
Skrjabin, K. I., 114
Slater, E. C., 153, 236
Sleeth, B., 452
Slein, J. B., 92
Slie, R. B., 227
Slotnick, I. J., 255, 256
Sluvko, A. L., 386
Smart, K. M., 503
Smiley, K. L., 267
Smirnova, N. P., 402
Smirnovskaia, A. S., 387
Smith, B. S. W., 168, 171, 177, 178
Smith, C. R., 535
Smith, D. C., 530
Smith, E. E. B., 161, 162
Smith, F. F., 461, 462
Smith, H., 77-102; 77, 78, 79, 82, 83, 84, 85, 87, 88, 89, 90, 91, 92, 93, 95, 96, 97
Smith, J., 303
Smith, J. D., 257, 258
Smith, J. L. K., see Kirby-Smith, J. L.
Smith, J. M., 502
Smith, J. W., 56
Smith, K. M., 442, 443, 444, 445, 449, 454
Smith, L., 154
Smith, M. E., 53, 54, 55, 60
Smith, M. H. D., 539
Smith, M. L., 493
Smith, M. M., 496
Smith, N., 86
Smith, P. F., 249, 250
Smith, P. W. H., see Harris-Smith, P. W.
Smith, R. A., 181, 183, 184, 249
Smith, R. R., 58
Smith, T., 136
Smith, W., 58, 419, 495, 496
Smith, W. E., 18
Smith, W. J., 419
Smith, W. L., Jr., 424
Smorodintsev, A., 399
Smorodintsev, A. A., 405
Sneider, U. C., 272
Snell, E. E., 253, 266, 267, 272
Snell, F. D., 534
Snell, N., 267
Snellbaker, L. F., 56
Snyder, H., 113
Snyder, J. C., 63
Sobel, G., 61, 64
Soberheim, G., 87, 88, 89
Sobotka, H., 251, 289, 290
Socolofsky, M. D., 314
Sodeman, W. A., 106
Sokatch, J. T., 147, 150, 154, 164
Sokurova, E. N., 391
Solov'ev, V. N., 401
Sommerville, R. G., 67
Somner, H., 81, 96
Sondheimer, E., 417
Sonneborn, T. M., 310
Sordelli, A., 533
Šorm, F., 258
Southam, C. M., 58
Southcott, B. A., 253
Souza, P. de, 423
Sowden, F. J., 176
Sowden, J. C., 148
Spada-Sermonti, I., 346
Spárek, J. V., 310
Speck, M. L., 251, 266
Spector, B. K., 106
Spencer, D., 216, 226, 227, 232
Spencer, M. J., 503
Spiegelman, S., 5, 6, 10, 13, 15, 16, 259, 312, 347, 350, 368, 370
Spilman, W., 347
Spink, W. W., 496
Spirin, A. S., 389
Spirites, M., 535
Spitz, S., 110
Spizizen, J., 5, 7, 11, 347
Spoehr, H. A., 303
Spoerl, E., 271
Spooner, D. F., 108
Sprinson, D. B., 253
Srb, A. M., 375
Sreenivasan, A., 212
Stackiw, W., 67
Stadler, D. R., 371, 372
Stadtman, E. R., 149
Stähelin, H., 4, 5, 9, 12
Stahl, F. W., 27, 40, 42, 43, 324
Stakman, E. C., 377
Stallones, R. A., 51, 52, 59, 65
Stambaugh, W. J., 430
Stamm, W. P., 103
Stanier, R. Y., 1, 160, 167, 168, 191, 192, 194, 212, 259, 356
Stanley, J. L., 79, 82, 83, 88, 89, 90, 91, 92, 97
Stapleton, G. E., 508, 511, 512
Stark, O. K., 531
Starkey, R. L., 429, 432, 434
Starlinger, P., 349

Starr, G. H., 419
Starr, M. P., 325
Starr, P. B., 542
Starr, T. J., 301
Stauffer, J. F., 374, 375
Stebbins, M. E., 297, 301
Steckelmacher, S., 114
Stedman, R. L., 535
Steel, R., 155, 156
Steenson, T. I., 177
Stehle, R. L., 543
Stein, J. H., 88
Stein, J. R., 296
Stempen, H., 18
Stent, G., 321
Stent, G. S., 6, 10, 27, 38, 39, 40, 42, 43, 322, 366
Stern, C., 368
Stern, J. R., 180
Sterne, M., 79, 325
Stessel, G. J., 425
Stevens, H. M., 219
Stevens, M. F., 493
Stevenson, R. E., 52
Steward, F. C., 469, 474, 475
Stewart, F. C., 456
Stewart, M. T., 51
Steyskal, F., 533, 534
Steyskal, K., 533, 534
Stich, H. F., 351
Stiefel, J. R., 535
Stiemie, S. F., 452
Stirewalt, M. A., 116
Stjernholm, R., 165, 179
Stockdill, S. M., 235
Stocker, B. A. D., 310, 335, 351
Stoeber, F., 260
Stoffel, P. J., 270
Stokstad, E. L. R., 267, 296
Stolmakova, A. I., 404
Stone, B. A., 160
Stone, R. L., 532
Stone, R. W., 173, 174
Stoner, H. B., 95
Stonier, T., 478
Storck, R., 4, 6, 7, 10, 16
Storey, I. D. E., 161
Storm, J., 297
Stoy, V., 221
Strain, H., 303
Strandstrom, H., 55, 60, 61, 64
Strange, R. E., 4, 9, 28, 92, 163
Straub, J., 376
Straughn, W. R., 217
Strawinski, R. J., 173
Strecker, H. J., 179
Streisinger, G., 40, 42
Strittmatter, C. F., 261
Strominger, J. L., 20, 34, 163
Strong, F. C., 418
Strong, F. M., 475
Struckemeyer, B. E., 480
Stuart, L. S., 541
Stuart-Harris, C. H., 58, 59
Stubbs, J., 425, 431
Stulberg, C. S., 98
Stunkard, H. W., 117
Su, S., 114
Sukhov, K. S., 385, 387
Sukhova, M. N., 400
Sulzbacher, W. L., 518
Sunakawa, S., 38, 39
Süpfle, K., 534
Surgalla, M. J., 95, 494, 520
Surikova, E. I., 392
Surkiewicz, B. F., 534, 542
Suskind, R. G., 51, 52, 58, 61, 64, 65
Suskind, S. R., 353, 354, 369
Sussman, A. S., 365, 370
Sutter, D., 263
Sutton, C. R., 260
Sutton, M. D., 422
Sutton, W. B., 190
Suvorova, G. V., 404
Suyama, Y., 369
Suzuki, T., 67
Svedmyr, A., 52, 56, 57, 58, 61, 64
Swanson, H. S., 50, 63
Sweeney, B. M., 297
Swellengrebel, N. H., 2
Swift, H. H., 312
Swim, H. E., 184
Sykes, G., 536
Sylvester, R. F., 504
Symonds, K. V., 177
Syrett, P. J., 207
Syverton, J. T., 98
Szidat, L., 117
Szilagyi, D. E., 544
Szilard, L., 317
Szkolnik, M., 417, 418, 428
Szulmajster, H. de R., see Robichon-Szulmajster, H. de
Szybalski, W., 324, 346, 358

T

Tabachnick, J., 191, 192
Tabak, H. H., 527
Taber, W. A., 250
Tabor, C. W., 268
Tabor, H., 268
Tager, M., 495, 497, 498, 499
Takacs, W. S., 535
Takahashi, H., 203-46; 209, 216, 218, 222, 226, 232, 234, 270
Takahashi, S. M., 542
Takahashi, T., 366
Takeda, Y., 212
Talling, J. F., 302
Tamura, G., 265
Tanabe, H., 117
Tanaka, C., 60
Tanaka, N., 66
Tang, P., 221
Taniguchi, S., 209, 211, 215, 218, 221, 223, 226
Tanner, F. W., Jr., 254
Taranenko, A. F., 398
Tarizzo, M. L., 63
Tarr, H. L. A., 253
Tatum, E. L., 167, 168, 170, 365, 370
Taubeneck, U., 18
Tausson, W. O., 173, 174
Tavormina, P. A., 262
Taylor, A. L., 511
Taylor, A. R., 52
Taylor, A. W., see Wilson Taylor, A.
Taylor, D. J., 104
Taylor, E. S., 535, 536
Taylor, H. E., 326
Taylor, H. E., see Ephrussi-Taylor, H.
Taylor, J. H., 321
Taylor, R. M., 107
Taylor, W. W., 527
Teas, H. J., 365, 370
Tebbutt, A. H., 492
Teixeira de Freitas, J. F., 114
Tempest, D. W., 79, 88, 90, 91, 92
Teramoto, S., 265
Terekhova, N. A., 398
Terry, L. L., 105
Terwilliger, D. E., 320
Tessler, J., 541
Thal, E., 95
Thatcher, F. S., 494
Thayer, P., 296
Thiessen, C. P., 185
Thoma, R. W., 263
Thomas, C. A., Jr., 39, 43, 322, 347
Thomas, C. H., 429
Thomas, E. H., 105
Thomas, E. P., 114
Thomas, H. E., 444, 455, 456
Thomas, H. R., 415, 416, 419
Thomas, J. L., 251
Thomas, L., 94
Thomas, L. J., 114, 117, 118
Thomas, L. R., 58
Thomas, P. T., 375
Thomas, R., 328
Thompson, J. B., 422
Thompson, P. E., 104, 105
Thompson, R., 20
Thompson, T. G., 288
Thorne, G. F., Jr., 426
Thorne, G. W., Jr., 426

Thornley, M. J., 508, 509, 518, 520
Thornton, H. G., 173, 177
Thorp, F., 141, 535
Thorogood, E., 236
Thorsson, K. G., 3, 11, 16, 52, 56, 57, 58
Thunberg, T., 179
Thygeson, P., 51, 61, 62, 63, 64
Timokhina, M. I., 402
Tissières. A., 153, 236
Titmuss, D. H. J., 98
Tobie, J. E., 103, 105
Todd, W., 111
Tokhver, V. I., 237, 384
Tolbert, N. E., 181
Tolchinsky, E., 528
Tolmach, L. J., 27, 37, 330, 333
Tomarelli, R. M., 254
Tomcsik, J., 2, 3, 4, 6, 11, 85, 91
Tomizawa, J., 38, 39
Tomlinson, A. H., 251, 265
Tomlinson, N., 179, 184, 251
Tompsett, R., 495
Tongeren, H. A. E. van, 541
Tonney, F. O., 106
Toomey, J. A., 535
Toothhell, J. P. R., 432
Topley, W. W. C., 493
Topper, Y. J., 166
Torrey, J. G., 483
Toumanoff, C., 114
Tournier-Lasserve, C., 115
Tousimis, A. J., 52
Townsley, P. M., 263
Tracey, R. L., 533
Trápido, H., 112
Traum, J., 140
Travis, R. V., 461
Traxler, R. W., 267
Trecanni, V., 173, 174
Treffers, H. P., 88
Trelawny, G. S., 272
Trumball, M. L., 110
Tsui, T., 475
Tsukamura, M., 228
Tuck, H. A., 60, 61, 64
Tulasne, R., 17, 18
Tumanian, M. A., 391
Tung, M., 114
Turel, F., 486, 487
Turner, D. I., 432
Turner, E. M., 251
Turner, G., 535
Turner, H. C., 51, 58, 59
Turner, J. C., 167
Tuve, T. W., 258
Tyrrell, D. A. J., 58, 61

U

Uchitel', I. I., 402
Uehleke, H., 18
Uetake, H., 339
Ukita, T., 270
Umbarger, H. E., 187
Urbain, W. M., 517
Uribe, C., 107
Ushakova, A. A., 403
Utter, M. F., 179
Uyeno, S., 247
Uzdins, K., 533

V

Vahle, C., 2
Vainberg, B. G., 387
Vakili, N. G., 378
Valentine, F. C. O., 495
Valleau, W. D., 449
Vallentyne, J. R., 302
Van den Ende, J., 543
van den Hamer, C. J. A., see Hamer, C. J. A. van den
Van der Meer, G., 110
van der Veen, J., see Veen, J. van der
Vanderwinkel, E., 4, 6, 7, 10
Vande Velde, A. J. J., 533
Vanecko, S., 225, 226
Vanek, J., 110
van Herick, W., see Herick, W. van
van Heyningen, W. E., see Heyningen, W. E. van
van Iterson, W., see Iterson, W. van
Vaniushin, B. F., 389
Van Lanen, J. M., 254, 478
Van Niel, C. B., 213, 215, 216
Van Phung, Le, see Le-Van Phung
VanRavenswaay, T., 52, 56, 57
Van Schaack, V., 424
Van Sumere, C. F., 164
van Tongeren, H. A. E., see Tongeren, H. A. E. van
Van Uden, N., 249
Varner, J. E., 225, 234, 235
Vasconcelos, C. M., 115
Vasilenko, A. G., 385
Vasilov, S. I., 406
Vasiurenko, K. I., 389
Vaughn, C. M., 117
Vaughn, J. R., 423, 428
Vaughn, R. H., 191, 192
Veen, J. van der, 54, 58, 63
Veisfeiler, I. K., 387
Vely, V. G., 209
Vening, L. D., 250
Venkataraman, R., 212
Vennes, J. W., 3, 6, 16, 17
Vergara, C., 418
Verhoeven, W., 208, 212, 213, 215, 216, 223
Verlinde, J. D., 60
Vernon, L. P., 214
Verona, O., 431
Vershilova, P. A., 405
Versteeg, J., 60
Vigneaud, V. du, 269
Vinegar, R., 527
Vinh, L. T., 50, 63
Vinograd, J., 324
Viranuvatti, V., 114
Virat, J., 50, 63
Virtanen, A. I., 203, 216, 225, 237
Visconti, N., 42
Vishniac, H. S., 284
Vishniac, W., 146
Visser, D. W., 270
Viswanathan, L., 266
Vivell, O., 62
Vivino, J. J., 496
Vlppö, A., 110
Vogel, H., 116, 117, 118
Vogel, H. H., 480
Vogel, H. J., 179, 257, 350, 353
Vogel, J., 68
Vogt, M., 529
Voight, R. B., 528
Vojnovich, C., 254
Volcani, Z., 486
Volkin, E., 41
Vollenweider, R. A., 283
Vollmayer, E., 270
Von Brand, T., 108
von Glander, R., see Glander, R. von
Von Plotho, O., 533
Von Saltza, M. H., 475
Vulton, M. W., 431
Vygodchikov, G. V., 400, 403

W

Wachsman, J. T., 16
Wagenaar, R. O., 520
Wager, O., 55
Wagner, R. P., 365, 370
Wahlroos, Ö., 209
Wailling, D. G., 496, 499, 504
Wainwright, L. K., 317
Wainwright, S. D., 206, 210, 217
Waite, C. P., 543
Walker, D., 61, 64
Walker, D. L., 66, 67
Walker, J. B., 287
Walker, J. C., 461, 482
Walker, J. T. A., 529
Walker, N., 167, 173, 174, 177

Walker, T. K., 155, 156
Walker, W. J. G., see Greaves-Walker, W. J.
Wallace, J. M., 451, 452
Wallen, V. R., 427, 434
Wallendal, P., 487
Wallis, M., 263
Walston, H. D., 496
Walston, V. M., 105
Walton, B. P., 257
Ward, E. N., 528
Ward, R., 51
Ward, T. G., 50, 51, 52, 53, 54, 55, 59, 60, 61, 62, 63, 64, 65
Wardlaw, A. C., 111
Ware, G. C., 230
Warfield, M. S., 51, 52, 59, 65
Warner, A. C. I., 206
Warren, J., 53
Warshaw, L. J., 543
Warshowsky, B., 525-50
Washington, J. E., 105
Washington, O., 339
Watanabe, A., 237, 291
Watanabe, I., 40
Waters, C. E., 442
Watertor, J., 482
Watson, D. W., 77, 82, 87, 88, 89, 90, 91, 94, 95
Watson, I. A., 378
Watson, J. D., 30, 38, 41
Wattenberg, H., 287
Weaver, R. H., 251
Weber, M. M., 153
Weed, L. A., 532
Weibull, C., 1-26; 1, 2, 3, 4, 6, 7, 10, 11, 12, 13, 14, 16, 17, 18
Weidel, W., 27-48; 20, 28, 29, 30, 31, 32, 33, 34, 36, 37, 41
Weigle, J. J., 336, 338
Weil, E., 81, 82, 87, 88, 91
Weimberg, R., 184
Weinberger, H. J., 17, 18
Weindling, R., 415, 416
Weiner, M., 310
Weinfeld, H., 256
Weinstein, A., 371
Weinstein, P. P., 115
Weintraub, R. L., 270
Weissman, N., 82, 88, 90
Welch, A. D., 252
Weller, T. H., 115
Wells, A. F., 203
Wells, A. Q., 86
Wells, W. H., 117
Welsch, M., 8
Welshimer, H. J., 3
Wender, I., 174
Werkman, C. H., 179, 267
Werner, J. H., 50, 51, 52, 53, 55, 58, 63
Werner, R., 534
Wernicke, R., 533
Wessel, G., 222
Wester, R. E., 421
Westerfeld, W. W., 222, 228
Westergaard, M., 318, 319, 336
Westlake, D. F., 302
Westphal, A., 106, 110
Westwood, J. C. N., 98
Wetmore, R. H., 474, 477
Wheeler, H. E., 365-82; 372, 376
Whistler, R. L., 163
White, A. G. C., 105
White, G. B. B., see Bruce White, G. B.
White, G. F., 112
White, P. R., 469, 470, 472, 477, 478, 485
Whitehead, J. E. M., 503
Whitehouse, H. L. K., 372
Whiteside, J. E., 51, 58, 59
Whiting, B. L., see Lee-Whiting, B.
Whitwell, F., 93
Wiame, J. M., 4, 6, 7, 10
Wickerham, L. J., 224
Wijler, J., 213, 216
Wilcoxson, R. D., 377
Wild, G. M., 164
Wilder, H. C., 113
Wilkins, M. H. F., 366
Wilkinson, J. F., 166
Williams, A. L., 267
Williams, H. H., 258
Williams, L. E., 422, 427
Williams, N. J., 512, 514
Williams, O. B., 541
Williams, R. C., 35
Williams, R. E. O., 493
Williams, R. P., 353
Williams, W. L., 137
Williamson, D. H., 21
Willison, R. S., 455, 457
Willson, C. P., 187
Wilson, A. T., 541, 542
Wilson, G. B., 375
Wilson, G. S., 82, 94, 98, 493
Wilson, J. F., 112
Wilson, P. W., 153, 203, 230, 231, 235, 236, 237
Wilson, R. A., 416, 418
Wilson, S. M., 314
Wilson Taylor, A., 81, 97
Wilson, T. G. G., 236
Wilson, W. B., 287, 297
Wiltshire, G. H., 173, 174, 177
Winfield, M. E., 231
Wingard, S. A., 448
Winge, Ø., 366, 367, 370
Winogradsky, S., 160
Winter, G., 291
Winter, H. F., 416
Wirth, A., 114
Witenberg, G., 114
Withell, E. R., 536
Witkin, E. M., 311, 312, 313
Woke, P. A., 109
Wolfe, M., 222
Wolfe, P. A., 103
Wolfe, R. S., 179, 255
Wolff, R. L., 271
Wolff, S., 313
Wolff, S. M., 269
Wolfin, E. F., 511, 513, 514, 518
Wolin, E., 15
Wolin, E. F., 521
Wolin, H. L., 250
Wollman, E. L., 27, 42, 43, 44, 45, 337, 338, 340, 341, 342, 343, 344
Wong, D. T. O., 180, 181, 182, 183, 249
Wood, H. G., 179
Wood, T. H., 511
Wood, W. A., 145, 149, 164, 187
Woodbury, D. H., 511
Woods, D. D., 187, 189
Woods, P. S., 321
Woodward, G., 535
Woodward, V. W., 319, 369, 374
Woody, H. B., 107
Woody, N. C., 107
Wooley, J. T., 222
Woolf, A., 113
Wooliams, G. E., 420
Woolridge, R. L., 51, 54, 58, 59, 60
Work, E., 3, 5, 9, 10, 18, 163, 271
Worth, C. B., 112, 113
Wosilait, W. D., 153
Wray, A. M., 425, 431
Wright, E. S., 536
Wright, G. C., 92
Wright, H. B., 155
Wright, H. D., 95
Wright, J. M., 425, 431
Wright, L. D., 257
Wright, W. H., 103
Wyatt, J. R., 110
Wyss, D., 525, 530, 537
Wyss, O., 235, 236, 314, 319

Y

y Abbonenc, C., see Abonnenc, C. y
Yaeger, R., 108
Yagi, T., 227
Yamada, M., 66
Yamada, T., 216
Yamagata, S., 223
Yamamoto, Y., 67
Yamane, I., 266
Yanofsky, C., 252, 354, 369,

370
Yant, W. P., 543
Yarbrough, H. F., Jr., 254
Yasui, G., 509
Yasunobu, K. T., 168
Yates, M., 78
Yates, R. A., 261
Yčas, M., 258, 259, 261, 273
Yesair, J., 541
Yogore, M., 114
Yokoyama, H., 262
Young, D. A., 487
Young, H. C., 416
Young, M. Y., 533
Younger, J. S., 528
Yudkin, J., 533
Yura, T., 348, 349
Yuvilai, A., 271

Z

Zaiman, E., 58
Zaiman, T. E., 61
Zaitseva, G. N., 388
Zakharova, I. I., 391
Zakstel'skaia, L. I., 405
Zamenhof, S., 316, 334
Zarudnaya, K., 535
Zasukhina, G. D., 400
Zatulovskii, B. G., 392
Zaumeyer, W. J., 415-40; 415, 416, 419, 420, 421, 425, 431, 434
Zavarzin, G. A., 229, 391
Zeeuw, D. J. de, 423
Zehender, C., 18
Zehnder, A., 302
Zeitlenok, N. A., 406
Zelle, M. R., 311, 346, 365, 508, 511, 512
Zemskov, M. V., 396
Zetterberg, B., 61, 64
Zhdanov, V. M., 405
Zherikova, A. D., 404
Zhmaeva, Z. M., 399
Zhukova, I. G., 4, 7, 10, 14, 390
Zichis, J., 535
Ziffer, J., 421, 425
Zil'ber, S. A., 400, 401
Zimenko, T. G., 394
Zimmerman, L. N., 258
Zinder, N. D., 5, 7, 11, 34, 42, 335, 338, 344, 346
Zintz, R., 62
Zubok, L. B., 391
Zuckerman, B., 419
Zucker, M., 223, 226
Zuelzer, W., 113
Zwartouw, H. T., 77, 78, 79, 84, 85, 88, 91

# SUBJECT INDEX


A

Absorption of antibiotics by plants, 432
Acetobacter
  celluloseless mutants of, 155-56
  enzyme preparations from, 157-59
  metabolism of, 155-56
Actokinase, 148
α-Acetolactate
  formation of, 187
Actidione
  control of cherry leaf spot by, 417
Actinoidin, 392
Acute conjunctivitis, 62-63
Acute febrile pharyngitis, 61-62
Acute respiratory disease, 58-60
Adaptation
  to nitrate reduction, 217
Adaptive enzyme synthesis
  genetic control of, 353-54
Adenosine triphosphate
  net gain in fermentations, 150
Adenovirus illnesses
  miscellaneous factors concerning, 63-65
Adenovirus pneumonia, 63
Adenoviruses, 49-58, 384
  characterization of, 51-52
  clinical syndromes induced by, 50
  epidemiological studies on, 50
  hemagglutination by, 56
  host range of, 52-53
  infectivity titers of, 53-54
  inhibitors against, 54
  interaction with tissue culture cells, 56-58
  oncolytic effects of, 58
  serological tests with, 54-56
  serotypes of, 50-52
  size of, 52
  stability of, 52
  vaccines against, 65
Adsorption of bacteriophage, 27-48
Aggressins, 78
  of Bacillus anthracis, 91
  tests for, 86
Air
  disinfection of, 538-39
Alcohols, polyhydric
  metabolism of, 186-87
Algae
  antagonisms between, 302-3
  nitrogen fixation by, 291
  nitrogen requirements of, 206-7
  nutrition and ecology of, 279-308
Algal groups
  distinctive nutritional features of, 281
  influence of temperature on, 290-91
  major elements required by, 286-87
  nitrogen requirements of, 291
  phosphorus requirements of, 292-95
  plant hormone requirements of, 295-301
  trace element requirements of, 287-90
  vitamin requirements of, 295-301
Alkylating agents
  as disinfectants, 540
Amebiasis
  diagnosis of, 105
  treatment of, 105-6
Amebic dysentery
  and host nutrition, 104
  susceptibility to, 104
Amino acids
  essential, 250
  interrelationships in growth, 268
  utilization of D-isomers of, 251
Ammonia
  assimilation of, 204
  incorporation in amino acids, 208
  permeability of bacteria to, 204
Ammonia assimilation
  fungal products of, 207-8
  inhibition of, 234
Ammonia utilization
  influence of tricarboxylic cycle acids on, 206
Ammonium dehydrogenase, 226
Anergie, 473
Angular leaf spot of cucumber
  control of, 422
Animal diseases
  control of, 127-44
  epizootiology of, 127-29
  eradication in the U. S., 129-42
  miscellaneous, need for control of, 143
  U. S. laws for control of, 129
Antagonism
  between algae, 302-3
  and plant infections, 415
Anthracnose
  control of, in bean, 421
Anthranilic acid
  tryptophan synthesis and, 252
Anthrax
  basis of immunity to, 91-92
  factors influencing development of, 87-93
  fatal syndrome in, 88-91
  major pathologic changes in, 89
Anthrax toxin, 88
  chemical nature of, 90
  discovery of, 89-90
Antibiotics
  control of plant diseases by, 415-40
  mode of action in plant diseases, 431-35
  Russian literature on, 392-93
  see also individual agents
Antibody formation
  Russian literature on, 401-3
Antigenic structure
  influence of prophage on, 339
Antigens
  properties of, 389
Antiseptic activity of mercurials, 532
Apple rootstock disorder, 453
Apples
  diseases, control of, 416
Aromatic compounds
  metabolism of cleavage products of, 168-71
  miscellaneous, metabolism of, 171-78
  oxidation of, 167-78
  pathways of degradation of, 169
  ring cleavage of, 167-68
Ascohyta blight of peas
  control of, 423
Autolysis of bacteria, 8
Auxins
  in plant tissue cultures, 472

Axenic development of nematode parasites, 115

B

Bacillus anthracis
in vivo metabolism of, 92
properties of in vivo-grown, 82-83
virulence factors of, 78, 87-93
Bacillus anthracis toxin
production of, 93
Bacteria
immunological properties of structures of, 17
isolation of in vivo-grown, 82-86
pathogenicity of in vivo-grown, 77-102
Bacterial blights of beans
control of, 419-20
Bacterial blight of celery
control of, 421
Bacterial genetics, 309-64
see also Mutation; Replication; Transformation; Transduction; Lysogenic conversion; Conjugation; Heterokaryosis; and Genes
Bacterial protoplasts, 1-26
see also Protoplasts
Bacterial spot
of peaches, control of, 417
of tomatoes and peppers, control of, 424
Bacterial viruses
see Bacteriophage
Bacterial wilt of corn
control of, 427
Bactericidal action of radiation, 510-11
Bactericidal agents
action of, 391
Bacteriophage
adsorption and penetration of, 27-48
attachment and penetration mechanisms, 34-35
carriage of genetic information by, 37-38
functional anatomy of, 34-38
genetic recombination in, 43
genetics at the molecular level in, 42-44
germinal substance of, 38-41
maturation of, 40
penetration enzymes of, 31-34
production in protoplasts, 8
reviews on, 27
Russian literature on, 384
Bacteriophage pyrimidines
biosynthesis of, 256
Bacteriophage receptors
distribution and frequency of, 30-31
BCG vaccination, 405
Beans
control of blights of, 415, 419-20
Biology of microorganisms
Russian literature on, 388-95
Biosynthesis
feed-back control of, 269
Bird plague virus
nature of, 385
Black rot of rutabaga
control of, 422
Blood flagellates
epidemiologic patterns of, 107
Blood flukes, 115-17
Blue mold of tobacco
control of, 426-27
Bovine brucellosis, 138-39
Bovine tuberculosis, 137-38
Brassica viruses, 448
Broccoli
control of downy mildew of, 422
Brown rot of peaches
control of, 418
Brucella
effect of cellular products on virulence of, 97
Brucellosis, 397
bovine, 138-39

C

Cabbage black ringspot, 448
Calcium
role of, in nitrogen fixation, 235
Canker, bacterial
control of, 417
Carbohydrates
metabolism of, 145-202
Carbon dioxide
assimilation of, 389
Catalase
adaptive nature of, 261
Cattle
contagious pleuropneumonia of, 130
Cauliflower mosaic, 448
Celery blight
control of, 421
Cellulase
factors controlling production of, 161
induction of, 260
Cellulose
biosynthesis of, 264
breakdown of, 160-61
enzymes for degradation of, 160-61
inhibitors of, 157
site of formation of, 159
synthesis of, 155-60
Cell wall
of bacteria, nature of, 2
diaminopimelic acid and integrity of, 271
Cell walls
binding of bacteriophage by, 28
Cereal diseases
control of, 427
Cereals
virus diseases of, 461
Cestodes, 117-18
Chelating agents
growth of algae and, 288-90
Chemical composition of microorganisms, 389
Chemical disinfectants, 525-50
Chemosynthesis
Russian literature on, 389
Cherry diseases
control of, 417
Cherry rootstock disorder, 452-53
Cherry virus complex, 456-57
Chitin
biosynthesis of, 163-264
Cholera, hog, 142
Chrysanthemum bacterial wilt
control of, 429
Chrysanthemum stunt, 460
Cistrons, 43
Citramalate
formation of, 187-88
Citrovorum factor, 252
Citrus tristeza, 451-52
Coagulase-reacting factors, 497-99
Coagulase
staphylococcal, 495-99
Cobalamins, and algal growth, 301
Colitis, amebic, necessity of bacteria for, 103-4
Conjugation of bacteria, 340-46
direction of, 340-42
energy requirement for, 345
quantity of material transferred in, 342-46
role of tricarboxylic acid cycle in, 184-85
Conjunctivitis, acute, 62-63
Contagious pleuropneumonia of cattle, 130
Control of plant diseases, 415-40
Corn diseases

control of, 427
p-Cresol, oxidation of, 171
Croup-associated virus, 67-68
Crown gall, 477
Crucifers
control of diseases of, 422
Cucumber diseases
control of, 422-23
Cucumber mosaic, 448
Curly top of sugar beets, 450-51
Cutaneous larva migrans, 112-13
Cyanide, as a nitrogen-carbon source, 230
Cytochromes
of denitrifying bacteria, 213-15
nitrate influence on concentration of, 214-15
and nitrate reduction, 211
Cytoplasmic inheritance, 373-74
Cytoplasmic membrane of bacteria, nature of, 16

D

2:4-D (2:4-Dichlorophenoxyacetic acid)
degradation of, 177
Death
criteria of, 526-29
reversible, 530
Decarboxylases
inducible nature of, 260
Denitrification, 208-28
influence of oxygen on, 213
intermediates of, 215-17
pathways of, 215-17
problems of, 218
Deoxyribonucleic acid
antigenicity of, 335
assay for, 272
as carrier of genetic specificity, 38-41
effect of ionizing radiation on, 334
effect of mutagens on, 333-34
heterogeneity of, and transformation, 332-34
incorporation of 5-bromouracil in, 316
influence of radiation on, 313-16
replication of, 38-39, 321-24
structure of, 38, 365
Detergents
toxic action of, 269
Diagnostic methods
Russian literature on, 406
Diaminopimelic acid
and cell wall integrity, 271
Dicarboxylic acids
synthesis of, 178-85
Diphasic variation, 355
Diseases, animal
control of, 127-44
Diseases, human
of protozoan origin, 103-12
Diseases, plant
antibiotic control of, 415-40
Disinfection, chemical, 525-50
criteria of death in, 526-29
test methods for, 529-30
Dodder latent mosaic, 447-48
Dourine, 137
Downy mildew of beans
control of, 421
Downy mildew of broccoli
control of, 422
Downy mildew of cucumbers
control of, 422
Dutch elm disease
control of, 430
Dysentery, 396

E

Early blight of tomatoes
control of, 425
Echinococcus miltilocularis, 117-18
Ecology of protozoa and algae, 279-308
Embden-Meyerhof pathway
energetics of, 146
Encephalitis viruses
resistance of, 392
Endotoxins
of Gram-negative bacteria, 98
Energetics of carbohydrate metabolism, 146-55
Energy transformations
review of, 155
Entamoeba histolytica
life cycle of, 104
metabolism of, 103-4
nutritional requirements of, 104
pathogenic characteristics of, 103-6
Enterotoxin
staphylococcal, 494
Entner-Doudoroff pathway
energetics of, 147
Enzymes
detection of activity in situ, 86
inducible, biosynthesis of, 259-61
localization in bacteria, 16-17
and penetration of bacteriophage, 31-34
Russian literature on, 391
staphylococcal, 495-501
Epidemic keratoconjunctivitis, 62-63
Epidemiological process, 395
Epizootiology of animal diseases, 127-29
Escherichia coli
receptor mosaic in cell wall of, 28-31
Ethylene oxide
disadvantages of use of, 543
equipment for use of, 542
mode of action of, 540-41
range of activity of, 541-42
sterilizing activity of, 540-43
Evolution of genetic material, 357-58
Exotoxin
staphylococcal, 491-93

F

Fatty acids
influence on growth, 262
toxic action of, 269
Fibrinolysin
staphylococcal, 499-500
Filaria larva migrans, 114
Filarias
reservoir of human, 114-15
Filterable forms
Russian literature on, 387
Fire blight
control of, 416
Flagella
Russian literature on, 385
systematic studies on, 1
Flagellates, human
epidemiological patterns of, 107
Flukes, 115-17
Food processing, radiation sources for, 508-9
Foods
radiation pasteurization of, 517-19
radiation preservation of, 507-24
reviews on, 508
required sterilization doses for, 516
Foot-and-mouth disease, 131-35
diagnosis of, 135
Formaldehyde
as a fumigant, 537-38
Formate
metabolism of, 188-89
Formate oxidizing enzyme, 189
Formic dehydrogenase, 188-89
Formic hydrogenylase,

188-89
Formylaspartic acid
inducible enzymes for, 260
Fruits, miscellaneous
control of diseases of, 418-19
Fumigation
agents for, 543
Fungi
genetics of, 365-82
genetics of sexuality in, 375-77
mutation of, 374-75
Fungi imperfecti
genetics of, 374

G

Galactoside-permease, 259
Gamones, 27
Gene conversion, 366-68
mechanism of, 367-68
Gene mutation
pleiotropic effects of, 356
Genes
control of protein specifity by, 350-55
interaction of, 355-56
linkage of, in bacteria, 311
nature of, 366
structure and function of, 368-70
Genetic control of metabolism, 370-71
Genetic information
of bacteriophage, 37
chemical theories concerning, 41-42
realization of, 39-40
Genetic material
evolution of 357-58
function of, 348-57
position and function of, 348-50
transfer of, 324-48
Genetics
of bacteria, 309-64
of fungal sexuality, 375-77
of fungi, 365-82
of imperfect fungi, 374
at a molecular level, 42-44
of phytopathogens, 377-78
of protoplasts, 19
Germicidal soaps, 536-37
Germinal substance of bacteriophage, 38-41
Germination
nutritional requirements for, 270
Glanders in horses, 131
Gliotoxin
and plant diseases, 415
Gluconate
streptococcal fermentation of, 147
Glucosan
synthesis of, 164
Glucose
inhibition of inducible enzyme formation by, 260
polymer formation, from pentoses, 163
stimulatory activity of degradation products, 266-67
synthesis of cellulose from, 158
Glutamic acid
accumulation of, 247-48
assay for, 271
fermentation of, 187-88
Glycerol
metabolism of, 186-87
Glycine
incorporation of, 248
Glycogen, bacterial
formation of, 164-65
Glycolipide
biosynthesis of, 264
Glycols
as air disinfectants, 539
Glyoxylate
metabolism of, 180
Glyoxylate cycle, 182, 248-49
Grain diseases
control of, 428
Grasses
control of diseases of, 428-29
virus diseases of, 461
Growth
amino acid requirements for, 250-52
carbon requirements for, 248-50
energy requirements for, 248-50
influence of lipides, sterols, and related compounds on, 262
inhibition of, 268-70
inorganic ion requirements for, 255
nitrogenous requirements for, 250-52
peptide requirements for, 250-52
of plant tissues, chemical regulation of, 475
requirements for, 248-68
stimulatory factors for, 266-68
synchronous, 314
unbalanced, and mutation, 316
utilization of fatty acids in, 262
utilization of purines, pyrimidines, and their derivatives for, 255-58
and vitamin utilization, 252-55
Growth factors, unidentified, 265
Growth inhibitors, miscellaneous, 270
Growth stimulating agents for plants, 474
Growth yields, 154-55

H

Halogens
as disinfectants, 537
Heat, and radiation
complementary effects of, 516-17
Helminths, producing human disease, 112-18
Hemadsorption viruses, 49, 68-69
Hemolysins
staphylococcal, 493
Herbicides
degradation of, 177
Heterokaryosis, 346-47, 372-74
Heterothallism, 376
Hiochic acid, 265
Hog cholera, 142
Homothallism, 376
Hop virus diseases, 461
Hops
control of downy mildew of, 429
control of mint rust of, 429
Hormones, sexual
in fungi, 375
Horses
glanders in, 131
Host factors
in resistance to staphylococci, 501-3
Host-parasite relationships
in staphylococcal infecfections, 501-3
Hyaluronic acid
synthesis of, 163
Hyaluronidase
factors controlling formation of, 165
staphylococcal, 500
Hydrazine
as intermediate in nitrogen fixation, 233
Hydrocarbons, polycyclic
metabolism of, 173
Hydrogen transfer by Mycobacterium lepraemurium, 83
Hydrogenase, 189
and nitrogen fixation, 231
Hydroxylamine reduction, 226-27
Hyponitrite reduction, 226-27

I

Immunity

to anthrax, 91-92
influence of irradiation on, 401
Immunization
against intestinal diseases, 404
Immunological properties of bacterial structures, 17
Immunology
Russian literature on, 400-6
Induced enzyme synthesis
resemblance to mutation, 310
Inducible enzymes
biosynthesis of, 259-61
control of, 259-61
glucose inhibition of formation of, 260
Infection of protoplasts, 347-48
Infectious diseases
Russian literature on, 395-400
Inflammation, 400
Influenza,
immunization against, 405
Influenza D virus, 66-67
Influenza virus
nature of, 385
Inorganic ions
growth requirements for, 255
Inorganic nitrogen metabolism, 203-46
Inositol
biosynthesis of, 254
utilization of, 254
Interaction of genes, 355-56
Interstitial plasmacell pneumonia, 110-11
Iron
influence of concentration on porphyrin synthesis, 263
influence on fermentation, 390
Irradiated foods
public health aspects of, 519-21
Itaconate
synthesis of, 185-86

J

JH virus, 70-71

K

Keratoconjuctivitis, epidemic, 62-63
Kinetin, 475

L

Lactic acid
metabolism of, 190
Lactobacilli
enzymic basis of homo- and heterofermenters, 149
Lactobacillic acid
synthesis of, 262
Larva migrans
cutaneous, 112-13
filaria, 114
spiruroid, 114
visceral, 113-14
Late blight of potatoes
control of, 424
Late blight of tomatoes
control of, 425
Leaf spot of cherry
control of, 417
Leishmania
nature of, 107-8
respiration of, 108
Leishmanias, 106-10
Leucocidin
staphylococcal, 494-95
Leuconostoc fermentation of glucose, 149
Leukemia
microbiological screening of chemicals against, 272
Levan
structure of, 164
L-forms of bacteria
and protoplasts, 17-19
Lignin
metabolism of, 174-77
Lipides
influence on growth, 262-63
Lipoic acid, 265
Logarithmic death rate of bacteria, 527-29
Lombard latent virus, 455
Lysis from without, 32
Lysogenic bacteria
incomplete strains of, 33
Lysogenic conversion, 337-40
Lysogeny
and transduction, 44-45
Lysozyme
action on bacteria, 2-9

M

Malaria
control of, 111-12
treatment of, 111
Malarial parasites, 111-12
Malate synthetase, 180
Man
parasitic diseases of, 103-26
Map distances, 43
Masked virus infection in plants, 441-68
Masking
definition of, 441
Maturation of bacteriophage, 40
Membrane transport, 247
Mercaptopurines
inhibition of synthesis by, 269
Mercurials
as disinfectants, 530-33
mode of action of, 531
Mesaconate
formation of, 187-88
Metabolic disturbances
and protoplast formation, 9
Metabolism
of Bacillus anthracis in vivo, 92
of carbohydrates, 145-202
energetics of, 146-55
genetic control of, 370-71
of polyhydric alcohols, 186-87
of polysaccharides, 155-67
reviews on, 145-46
Metabolites
reactivation of bacteria by, 527
Mevalonic acid, 265
Microbial nutrition, 247-78
Microbiologic assays, 271-72
Microorganisms
radiation resistance of, 511-16
Milieuchart, 282
Molar growth yields, 154-55
Molybdenum
and nitrate reduction, 218-23
in nitrogen fixation, 234-35
Morphology
effect of nutritional conditions on, 270-71
Mosaic diseases, 443-50
Motility
genetic factors influencing, 336
variation of, 310-11
Multiple sclerosis
as a virus disease, 400
Mumps, 399
Muramic acid, 163
Mutagenesis
chemical, 318-21
radiation, 318-21
Mutation, 309-21
back-mutation, 319
and cell division, 317-18
concept of, 316
fungal, 374-75
and gene loci, 318-21
influence of nucleic acid precursors on, 316
influence of postirradiation metabolism on, 312-16
influence of postmutagen treatment on, 320
nonchromosomal, 309-10
rate of, 318

spontaneous, nature of, 317
and unbalanced growth, 316
x-ray induced, 314-15
Mutation frequency
factors influencing, 315
Muton, 43
Mycobacterium leprae-murium
hydrogen transfer capacity of, 83
Mycobacterium tuberculosis
studies of in vivo-grown, 93-94
Myrobalan asteroid spot, 455
Myxoviruses
new, 65-69
properties of, 65

N

Naphthalene
metabolism of, 173-74
Nematodes, 112-15
axenic development in vitro of, 115
Nervous system
infections of, 395
role of, in immunology, 406-8
role of, in infectious disease, 406-8
Nitrate
kinetics of reduction of, 204
photochemical reduction of, 221
uptake of, 204-5
Nitrate assimilation, 208-28
effect of ammonia on, 205
reduction to nitrite in, 218-23
Nitrate reducing enzymes
adaptive nature of, 217-18
Nitrate reductase, 205-6, 209-11
Nitrate reduction
electron source for, 212-13
influence of oxygen on, 213
and phosphorylation, 209
types of, 208-50
see also Denitrification
Nitrate respiration, 208-28
in denitrifying organisms, 211-12
in nondenitrifying organisms, 209-11
Nitrate transport
adaptive nature of, 218
Nitrification, 228-30
Nitrifyers, 229
Nitrite reductase, 223
Nitrite reduction, 223-26
Nitrogen (inorganic)
absorption of, 204-8
metabolism of, 203-46
oxidation states of, 203
transport of, 204-8
Nitrogen fixation, 230-38
by blue-green algae, 291
cell-free, 230-32
evolutionary development of, 238
inhibitors of, 233-34
intermediates of, 232-33
role of metals in, 234-36
scheme for, 231
thermophilic, 384
ubiquity of, 236-38
Nitrogen-fixing organisms
metabolism of, 236
Nitrogen metabolism
general remarks concerning, 238-39
Nitrogen requirements of algae, 206-7
Nuclei
bacterial, 1
Nucleic acid synthesis
and mutation, 316
Nucleic acids
of bacteria, 388
incorporation of unnatural purines and pyrimidines in, 257-58
Nucleic acids, and proteins
interdependency of synthesis of, 259
Nutrition
microbial, 247-78
Nutrition of protozoa and algae, 279-308
Nutritional requirements of miscellaneous organisms, 250
Nutritional requirements of plant tissues, 472-85

O

Oak wilt
control of, 430
Oil
microbiology of, 393-95
Oil seed crop diseases
control of, 429
Oligodynamic action, 533
Oncolytic effects of adenoviruses, 58
Onion diseases
control of, 423
Organo-nitro compounds
reduction of, 227-28
Ornamental plants
viroses of, 460
Orotic acid
variation in enzymatic activity for, 261
Osmotic pressure of bacteria, 12
Oxalic acid
metabolism of, 190
Oxidation of aromatic compounds, 167-78
Oxidative phosphorylation, 152-54
Oxygen
influence of, on radiation resistance, 511

P

Pantothenic acid
assay for, 217
and related compounds, 253
Parasitic diseases of man, 103-26
Pasteurella pestis
studies of in vivo-grown, 95-97
Pasteurization
radiation and, 517-19
Pathogenic bacteria
isolation of in vivo-grown, 82-86
Pathogenicity
of bacteria grown in vivo, 77-102
of bacteria, reviews on, 77
biological tests for responsible compounds, 86-87
chemical basis of, 78-80
role of enzymes in, 495-501
of staphylococci, 491-506
Pathogenicity factors
in vivo conditions for production of, 80-82
Pea diseases
control of, 423
Peach diseases
control of, 417-18
Peach virus complex, 417-18
Pear diseases, control of, 416
Penetration of bacteriophage, 27-48
Penicillin
biosynthesis of, 264
induction of protoplast formation by, 15-16
mode of action of, 20-21, 34
Pentosans
hydrolysis of, 165-66
Pentose cycle
in gluconate fermentation, 149-50
Pentoses
fermentation of, 147-50
glucose polymers from, 163-64
Pepper diseases
control of, 424-25
Permeability of bacteria
review on, 11
Permeases, 193-94, 247, 259
Phagocytosis

Russian literature on, 401
Pharyngitis
acute febrile, 61-62
Pharyngoconjunctival fever, 60-61
Phenanthrene
metabolism of, 174
Phenol
derivatives of, and disinfection, 536
mode of action of, 536
Phenol coefficient, 529-30
Phenolic compounds
as disinfectants, 535-37
Philodendron leaf rot
control of, 430
Phosphoketolase, 148
Phosphorylation
coupling with electron transfer, 151-52
and nitrate reduction, 209
oxidative, 152-54
Photoreactivation, 320, 526
Photosynthesis
bacterial, 390
Phytopathogens
genetics of, 377-78
Picric acid
reduction of, 228
Piroplasmosis of cattle, 135-36
Plague
pathogenesis and immunity in, 95
Plague vaccine, 404
Plant diseases
antibiotics in control of, 415-40
bacterial, 486
description of, 384
fungal, 486
mode of action of antibiotics against, 431-35
nematode, 487
Plant tissue
isolation of, 470-71
Plant tissue cultures, 469-90
applications of, 487
modified morphology in, 472-83
normal, 470
physical conditions for, 471-72
physiology of, 472-83
single cell, 483-84
tumor, 470, 477-83
Plant virus diseases
antibiotic control of, 431
classification of, 442
symptomatology of, 442
Plant viruses
miscellaneous, 461
reservoirs of, 462
Plants
masked virus infection in, 441-68
Plasmolysis of bacteria, 2
Pleiotropic effects of gene mutation, 356
Pleuropneumonia of cattle, 130
Plum rootstock disorder, 453
Plum virus complex, 454-56
Pneumococci
virulence factors of, 97-98
Pneumocystic infection, 110-11
Pneumocystis carinii, 110-11
Pneumonia
adenovirus, 63
Poliomyelitis virus
inactivation of, 528-29
Polyamines
biosynthesis of, 268
nutritional importance of, 267
Polyhedric viruses
degradation and synthesis of, 385
electron microscopy of, 384
Polyhydric alcohols
metabolism of, 186-87
Polysaccharide synthesis
effect of nitrogenous compounds on, 165
Polysaccharides
metabolism of, 155-67
miscellaneous, 166
Porphyrins
influence of iron on, 263
Potato
abnormal graft reactions in, 453
Potato diseases
control of, 423-24
Potato mosaics, 443-45
Powdery mildew
control of, 420
Powdery mildew of wheat
control of, 428
Primary atypical pneumonia virus, 70
Prophage, 44-45, 337-40
Proteins
biosynthesis of, 258-59
replication function of, 39
Proteins, and nucleic acids
interdependency of synthesis of, 259
Protein specificity
gene control of, 350-55
Protocatechuic acid
breakdown of, 170
Protocatechuic oxidase, 167
Protoplasts
activity of, 390
bacterial, 1-26
bacteriophage reproduction in, 8
biochemical capabilities 10-11, 14
biological capabilities of, 10-11
criteria for, 5-6
effect of osmotic shock on, 15-16
effect of various enzymes on, 15
formation by ejection of, 4-5
genetic studies on, 19
of Gram-negative bacteria, 5
of Gram-positive bacteria, 2-5
infection by DNA of, 347-48
methods for preparation of, 6-10
osmotic function of, 11-14
permeability of, 11-14
permeases in, 194
relation to bacterial L-forms, 17-19
structure of, 2-6
subunits of degraded, 14-17
synthetic activities of, 14-15
yeast, 21
Prototrophs
factors influencing yield of, 312
Protozoa
nutrition and ecology of, 279-308
Protozoa producing human disease, 103-12
Prune dwarf, 455
Public health aspects of irradiated foods, 519-21
Purines
biosynthesis of, 257
as essential nutrilites, 255-58
Pyrimidines
as essential nutrilites, 255-58
synthesis of viral, 256

Q

Quaternary ammonium compounds
as disinfectants, 534-35

R

Rabies, 142
Radiation
doses for food sterilization, 516
and heat, complementary effects of, 516-17
lethal action of, 508
mechanism of bactericidal action of, 510-11
miscellaneous applications

of, 521
Radiation damage
recovery from, 312-16
Radiation preservation of food, 507-24
Radiation resistance of microorganisms, 511-16
Raffinose
fermentation of, 249
Raspberry mosaics, 445-47
Rate of death of bacteria, 527-29
Reactivation, 527
Receptor mosaic of Escherichia coli B, 28-31
Receptor substances, 27
Recons, 43
Replication, 38-39, 321-24
tracer studies of, 321-24
Resistance
nonspecific, 395
to staphylococci, 501-3
Respiration
and nitrogen fixation, inhibitors of, 233-34
theories of, 389
Respiratory syncytial viruses, 49, 69-70
Respiratory tract viruses
miscellaneous, 69-71
newly recognized, 49-76
Reversion
genetic, 319
Rhamnolipide
synthesis of, 166
Riboflavin
synthesis of, 254
Ribonucleic acid
participation in virus synthesis, 40-41
Rickettsiae
metabolism of, 391
Rickettsial diseases, 399
Ring rot of potatoes
control of, 424
Ringspot diseases, 448-50
Root rots
control of, 430-31
Rose virus diseases, 460
Russian literature
review of, 383-414
Rust of beans
control of, 421
Rust of wheat
control of, 427-28
Rutabaga
control of black rot of, 422

S

Safflower
control of damping-off of, 429
Salicylate
formation of, 173
Salk vaccine, difficulties encountered with, 528-29
Salmonella
variation in motility of, 310-11
Sanitizers, 535
Scab, cucumber
control of, 423
Scab, onion
control of, 423
Schistosome cercarial dermatitis, 117
Schistosome infections
control of, 116-17
Schistosomes, 115-17
Schizophrenia
as a virus disease 400
Scion-stock masked virus disorders, 452-53
Scrapie in sheep and goats, 141
Seed-piece decays of potato
control of, 423
Sendai (influenza D) virus, 66-67
Serologic methods
Russian literature on, 406
Serological tests
for adenoviruses, 54-56
Sesame
control of bacterial leaf spot of, 429
Shiro line-pattern virus, 455
Shwartzman reaction, 94
Silver
bactericidal preparations of, 533-34
as a disinfectant, 533-34
Single cell tissue cultures
plant, 483-84
Snapdragon rust
control of, 430
Soaps
germicidal, 536-37
Soft rot of spinach
control of, 424
Soil
microbiology of, 393-95
Sorbitol
metabolism of, 187
Soybean bacterial blight
control of, 429
Spinach
control of diseases of, 424
Spiruroid larva migrans, 114
Spores
formation and germination of, 270
Sporulation
nutritional requirements for, 270
Staphylococcal infections
general considerations of, 503-4
Staphylococci
factors determining pathogenicity of, 491-506
host factors in resistance to, 501-3
Stereospecificity of tartrate metabolism, 192
Sterilization
gaseous, 537-44
Sterilization by heat
stimulatory factors produced by, 267
Sterilizing doses of radiations, 516
Sterols
influence on growth, 262-63
Stone fruit viruses, 453-60
indexing hosts for, 458-60
Strawberry viruses, 462
Streptococci
factors associated with pathogenicity of, 94-95
products of in vivo-grown, 94
Streptomycin
control of bean halo blight by, 415
Streptomycin resistance
mutants for, 327-28
Structure of bacteria
reviews on, 1-2
Succinate
synthesis of, 179-84
Sugar beet curly top, 450-51
Sweet potato virus complex, 448
Symptomatology
of plant virus diseases, 442
Synchronous growth, 314, 324
Synthesis
requirements for, 248-68
Systematics, 384

T

Tapeworms, 117-18
Tartaric acid
inducible enzymes for, 192-93, 259
metabolism of, 190-93
T-1 bacteriophage
receptor for, 29
T-2 and -6 bacteriophages
receptors for, 29
T-3, -4, and -7 bacteriophages
receptors for, 28-29
T-5 bacteriophage
receptor for, 29-30
Temperature
influence on algal ecology, 290-91
Temperature coefficient of mutation, 318
Terregens factor, 265
Tetrad analysis, 371-72
Texas fever, 135-36
Thiamine

assay for, 271
penicillin derivatives as substitutes for, 255
Thymine-requiring bacteria
mutants of, 313
Tissue cultures
plant, 469-90
Tissue flagellates
epidemiologic patterns of 107
Tobacco diseases
control of, 425-27
Tobacco mosaic infections
control of, 431
Tobacco mosaic virus
development of, 385, 390
multiplication of, 485
Tobacco ringspot, 448
Tolerance
acquired, 401
Tomato diseases
control of, 424-25
Tomato ringspot, 449
Tomatoes
dodder latent virus in, 447
Toxin production
and prophage, 339
Toxins
pathogenic effects of, 396
Russian literature on, 404
staphylococcal, 491-95
see also individual toxins
Toxoplasma gondii, 108
Toxoplasmosis
development of causative organism of, 109-10
prevalence of, 109
Trace elements
and growth of algae, 287-90
Trace metals
assays for, 272
Transduction, 335-37
abortive, 335
and lysogeny, 44-45
unstable transductants in, 336
Transfer of genetic material, 324-28
Transformation, 325-35
assimilation of DNA during, 329-31
between different species, 326
competency in, 328-29
competition between DNA during, 330-31
models of genetic incorporation during, 332
and photoreactivation, 320
replacement phenomena in, 326
steps following initiation of, 331-33
time relationships in, 328-29
see also Deoxyribonucleic acid
Transforming principle
genetic heterogeneity of, 326-27
Translocase, 193
Translocation of antibiotics in plants, 432
Translocator, 193
Transport, 193-94
of nitrogenous compounds, 204-8
Transreplication, 368
Trees
control of diseases of, 430
Trematodes, 115-17
Tricarboxylic acid cycle
and bacterial conjugation, 185
and growth of yeast, 184
Tricarboxylic acid cycles, 178-85
Tricarboxylic cycle acids
influence on ammonia utilization, 206
Tristeza
of citrus, 451-52
Trypanosoma
respiration of, 108
Trypanosomes, 106-10
Tryptophan
biosynthesis of, 252
Tubercle bacilli
dispersion in tissues, 398
Tuberculosis
bovine, 137-38
Tularemia, 398
Tumors
antigens of, 403
plant, 398
Tumor tissue
of plants, 477-83
Tungstate
role of, in nitrogen fixation, 234

U

Uridine compounds
metabolism of, 162-63
Uridine pyrophosphoglycosyls
and polysaccharide synthesis, 161-62

V

Vaccine
against adenoviruses, 51, 65
Variation
Russian literature on, 385-87
Vegetables
control of diseases of, 419-25
Vesicular exanthema of swine, 140-41
Viral diseases
immunization against, 405
Russian literature on, 399-400
Viral diseases of plants
antibiotic control of, 431
tissue culture study of, 485
Virulence
in vivo factors influencing, 80-82
Virulence of bacteria
reviews on, 77
Virulence enhancing tests, 86
Virulence factors
of Bacillus anthracis, 78
and immunity, 79
of Pasteurella pestis, 95-97
Virus 2060, 70-71
Virus infections
masked, in plants, 441-68
Virus pyrimidines
biosynthesis of, 256
Viruses
animal and tissue culture-grown, 98
newly recognized respiratory tract, 49-76
reservoir of plant, 462
tissue culture of plant, 485
see also individual agents
Visceral larva migrans, 113-14
Vitamin $B_{12}$, 252-53
Vitamin requirements of algae, 295-301
influence of temperature on, 290-91
Vitamins
and microbial growth, 252-55

W

Walnut blight
control of, 419
Water
microbiology of, 393-95
Wheat diseases
control of, 427-28
White pine blister rust
control of, 430
Wildfire of tobacco
control of, 425-26
Wilt of cucumbers
control of, 422
Wilt of tomatoes

control of, 425

X

X-ray induced mutations, 314–15

Y

Yeast
irregular segregation in, 366
role of tricarboxylic cycle in growth of, 184

Z

Zygotic induction, 45